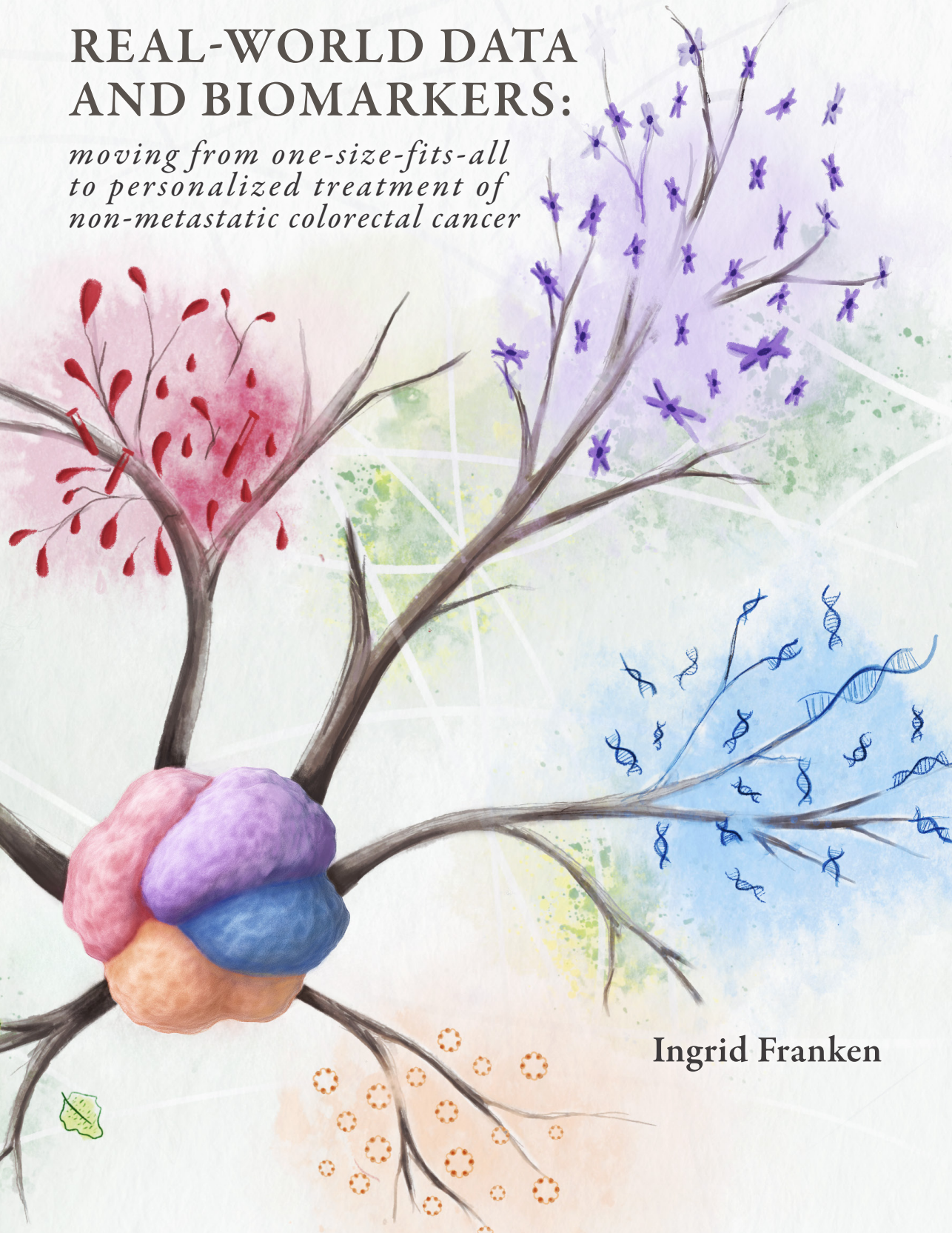
REAL-WORLD DATA AND BIOMARKERS:

moving from one-size-fits-all to personalized treatment of non-metastatic colorectal cancer



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Real-world data and biomarkers: moving from one-size-fits-all to personalized treatment of non-metastatic colorectal cancer

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Real-world data and biomarkers: moving from one-size-fits all to personalized treatment of non-metastatic colorectal cancer

Real-world data en biomarkers: van standaardbehandeling naar gepersonaliseerde zorg voor niet-gemetastaseerd colorectaal carcinoom
(met een samenvatting in het Nederlands)

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General introduction

Colorectal cancer (CRC) represents a major global health burden, ranking as the third most common cancer worldwide and the second leading cause of cancer-related deaths.[1] The Netherlands is among the top five countries with the highest incidence[2], with 9000 colon cancer (CC) and 3000 rectal cancer (RC) diagnoses in 2023.[3] Over half of patients are diagnosed with stage II or III CRC, based on the Tumor Node Metastasis (TNM) staging.[4] In stage II CRC, the tumor grows through the gastrointestinal wall (T3) or into surrounding organs (T4), but does not spread to lymph nodes (N0). In stage III CRC, up to three (N1) or more (N2) of the surrounding lymph nodes are involved. In both stage II and III CRC, there are no distant metastases (M0). In CC, resection of the primary tumor is the cornerstone of treatment and adjuvant chemotherapy (ACT) is recommended in stage III and high-risk stage II (pT4N0 pMMR). In non-metastatic RC, the indication for neoadjuvant (chemo)radiotherapy is largely based on TNM staging.[5]

In addition to TNM, guidelines recommend determination of proficient or deficient mismatch repair status (pMMR or dMMR, respectively), which is characterized by microsatellite stability or instability (MSS or MSI, respectively). dMMR tumors constitute a biologically distinct subgroup of non-metastatic CC (~15%)[6] and RC (2-5%)[7]. Different studies have shown that dMMR associates with better prognosis than pMMR in stage II colon cancer (CC), while its prognostic value in stage III CC varies. In rectal cancer (RC), the prognostic significance of dMMR is not well-defined.[8-10] dMMR is also prognostic of lower benefit from ACT, especially fluoropyrimidine monotherapy.[11,12] Instead, dMMR allows mutations and neoantigens to accumulate, causing sensitivity to immune checkpoint inhibition (ICI).[8] ICI shows promising results in randomized adjuvant studies in CC[13], as well as single-arm neoadjuvant studies in CC[14,15] and RC[16]. ICI has not yet become standard of care in non-metastatic dMMR CRC, only in the metastatic setting.

Current clinical practice and real-world data

In CC, standard of care consists of adjuvant chemotherapy (ACT) for patients with stage III or high-risk stage II disease. The type of ACT has remained the same since 2004, when the use of a fluoropyrimidine (5-fluoruracil or capecitabine) was combined with oxaliplatin (FOLFOX or CAPOX).[17,18] Since oxaliplatin is associated with increased toxicity, pMMR patients with contraindication or comorbidity may opt for

capecitabine monotherapy.[19,20] The IDEA trials showed lower toxicity and no clinically relevant inferiority in disease-free survival when reducing the duration of the oxaliplatin-containing doublet treatment from six to three months.[21,22] Non-inferiority was not statistically met for FOLFOX and for high-risk stage III (pT4/N2) disease, causing international guidelines to still recommend six months in those cases.[20,23] The Dutch guideline adopted in 2017 three months CAPOX for all patients, with quick and broad implementation in clinical practice.[5,24] This provides the opportunity to determine the impact on the general patient population, including relevant subgroups like high-risk stage III, using real-world data.

Real-world data (RWD) relates to all data on the patient health status and the delivery of health care collected from disease registries or electronic health records.[25] Such large data cohorts are valuable to provide information on older or less fit patients, who often do not meet the strict inclusion criteria of clinical trials.[26-28] Also for small subgroups like dMMR RC, RWD may help characterize response to standard of care to facilitate interpretation of the results of single-arm ICI trials. RWD of all new cancer diagnoses in the Netherlands is registered in the Netherlands Cancer Registry (NCR).[3] In addition, all patients with CRC can enroll in the observational Prospective Dutch Colorectal cancer (PLCRC) cohort, providing the additional informed consent and infrastructure to collect patient-reported outcomes, as well as tissue and blood for biomarker studies.[29] NCR and PLCRC therefore help translate treatment efficacy in trials to effectiveness in real-world clinical practice, and facilitate observational biomarker studies.

Need for biomarkers in CC

The one-size-fits-all recommendation of three months adjuvant CAPOX in high-risk stage II and stage III CC only benefits ~20% of patients. This group cannot be identified upfront, resulting in considerable mistreatment in the overall population. After resection of the primary tumor, half of patients are cured already and are thus overtreated with potentially toxic ACT.[30,31] Adjuvant treatment is aimed at the other half of patients, who have minimal residual disease (MRD) after surgery and depend on systemic treatment to be cured. However, ~30% of all patients experience recurrence despite resection and ACT, suggesting they are not sensitive to standard CAPOX and are undertreated. This highlights the urgent clinical need to improve selection of patients who are likely

to benefit from ACT, requiring a better understanding of who is not cured by surgery and who is likely to respond to CAPOX. To this end, biomarkers are required with prognostic value for recurrence and predictive value for response to treatment.

Biomarkers may include clinical, pathological or molecular (genomic, transcriptomic, proteomic) characteristics that are prognostic of (recurrence-free) survival and/or predictive of response to treatment. Dutch[5] and international guidelines[19,20,32] describe, in addition to stage and MMR, various biomarkers that are prognostic but not predictive (Figure 1). Clinical biomarkers include older age, right-sided tumor location[33], elevated carcinoembryonic antigen (CEA)[34], bowel obstruction, perforation and emergency surgery[35]. Conventional pathological risk factors include lymphatic[35,36] or vascular[37,38] or perineural[35,39] invasion, poor differentiation grade[40] and tumor budding[41,42].

Emerging tissue-based biomarkers in CC

In addition to conventional pathological characteristics, there is emerging evidence for the prognostic relevance of the tumor microenvironment (TME).[43] The TME includes all structures (i.a. vasculature and extracellular matrix) and non-malignant cells around the tumor cells, that can either suppress or support tumor development. Immune cells can infiltrate the tumor and recognize and respond to tumor-specific neoantigens, which are more prevalent in tumors with a high tumor mutational burden (in case of dMMR or POLE mutation). A higher density of T cells, quantified in the immunoscore, is associated with better prognosis.[44,45] On the other hand, a high amount of stromal cells like cancer-associated fibroblasts has been associated with poor prognosis.[46] The tumor-stroma ratio quantifies the percentage of stroma in the tumor based on a standard H&E slide, with >50% stroma as optimal cut-off for poor prognosis.[37,47]

On transcriptomic level, the tumor and TME have been described based on four main consensus molecular subtypes (CMS) of CC.[48] dMMR/MSI tumors largely correspond to the best prognostic CMS1, associated with strong immune activation and *BRAF* mutation. The larger pMMR/MSS subgroup largely subdivides into the epithelial CMS2 characterized by chromosomal instability and best oxaliplatin response[49], and the mesenchymal CMS4 with stromal invasion, poor prognosis and low benefit of 5FU[50]. The rarer metabolic CMS3 shows enriched *KRAS* mutation[9,48] and reduced CAPOX response in some[51] but not in other[52] studies. On genomic level, *BRAF* and *KRAS*

mutations are associated with worse prognosis after standard ACT.[53-55] But there is limited evidence supporting the ability of cancer driver mutations to predict benefit from treatment in the (neo)adjuvant setting[56-58] and to guide targeted treatment decisions like in the metastatic setting.[59-61] Therefore, these mutations are not standardly determined in the non-metastatic setting, and additional biomarkers are required.

Emerging liquid-biopsy-based biomarkers

One of the most promising biomarkers is circulating tumor DNA (ctDNA), which consists of DNA fragments shed by tumor cells to bodily fluids. ctDNA helps to non-invasively detect systemic presence of cancer cells. Thanks to the short half-life (15 min - 3 hours)[62,63], ctDNA detection is real-time and allows to monitor changes in response to neoadjuvant[16] and adjuvant treatment[64,65] of CRC. In the adjuvant setting, ctDNA may be employed to detect MRD after surgery in the form of micrometastases (0.2-2mm) that are undetectable by conventional clinical imaging but may progress to disease recurrence.[66-68] Multiple studies have shown that post-surgery ctDNA can be detected in a relatively small subset of stage II/III CC (~15%), but is associated with a very high recurrence risk (40-80%).[64-66,69] Therefore, presence of ctDNA may help select the patients who are not cured by surgery and in need of adjuvant treatment. However, ctDNA detection does not predict which treatment these patients would respond to.

Organoids provide an in vitro model to predict response

Functional *in vitro* models are required to study response mechanisms and help guide the development of novel therapies for resistant disease. Patient-derived organoids (PDOs) provide such a model system. PDOs are three-dimensional epithelial cultures that mimic the tumor they are derived from.[70-73] PDOs can be screened for sensitivity to drugs, both standard of care chemotherapy and alternative (targeted) treatment, to predict whether the corresponding patient is likely to respond.[74-76] Moreover, in case of resistance, PDOs can be used to identify alternative targets and develop novel therapie[77]. In addition, PDOs may be used as models to improve understanding of mechanisms underlying drug resistance and sensitivity. Most studies have been performed on palliative treatment of CRC metastases[76,78] or neoadjuvant treatment in RC[79,80]. Also in adjuvant treatment of CC, PDOs show promise to predict response to standard chemotherapy.[81,82]

Thesis aim en outline

This thesis focuses on patients with stage II–III CRC receiving standard of care one-size-fits-all treatment. The overarching aim is to identify patients who are over- and undertreated, and to inform personalized treatment strategies. The thesis is divided into two parts, as summarized in Figure 1.

The **first part** leverages real-world nationwide registry data from the NCR on the general CRC population, to describe clinical practice and to contextualize findings from trials.

In chapter 2, we aim to determine the impact of the guideline change from six to three months of adjuvant CAPOX on daily clinical practice. To this end, we compare overall survival between all patients with high-risk stage II/III CC treated with adjuvant CAPOX before (2015-2016) versus after (2018-2019) the guideline change. In addition, PLCRC provides access to patient-reported outcomes to gain insight into toxicity and quality of life. Together, this will provide more information on whether shorter CAPOX duration is safe and advantageous to reduce overtreatment.

We subsequently focus on smaller subgroups based on biomarkers, first the dMMR subtype of RC. Chapter 3 aims to provide context to emerging single-arm trials on ICI by describing real-world dMMR RC patients treated with current standard of care. To this end, we compare clinical and tumor characteristics, treatment approaches, and outcomes between all stage II/III dMMR and pMMR RC patients treated in the Netherlands.

The **second part** of the thesis investigates biomarkers that are prognostic of recurrence of CC after current standard adjuvant CAPOX (to identify who to treat) and predictive of response (to inform how to treat). To this end, tissue and blood are collected through PLCRC. Chapter 4-6 present results from the PLCRC-substudy PROVENC3, an observational study on ctDNA in stage III CC patients treated with ACT.

Chapter 4 introduces a novel tumor-informed whole-genome sequencing-based plasma circulating tumor DNA assay to detect minimal residual disease after surgery. We assess the prognostic value of post-surgery ctDNA for disease recurrence in stage III CC treated with ACT, as well as the added value to pT/N stage and MMR status.

In chapter 5, we aim to further refine risk stratification in stage III CC, by integrating ctDNA findings with the tissue-based pT/N stage and the tumor-stroma ratio. We combine these biomarkers to identify a very-low-risk group who may be overtreated with ACT, and a high-risk group who may be undertreated with CAPOX.

Chapter 6 involves more detailed genomic and transcriptomic profiling of stage III colon tumors, with the aim to identify predictive biomarkers for (lack of) sensitivity to CAPOX. To achieve this, we use post-surgery ctDNA detection to define a subgroup of patients who are not cured by surgery and depend on sensitivity to ACT to prevent recurrence.

In addition to observational clinical studies, we aim to model tumor-intrinsic resistance in patient-derived organoids in chapter 7. We perform CAPOX drug screens and correlate drug responses with genomic and transcriptomic features, to identify biomarkers predictive of treatment resistance and leads for alternative treatment avenues.

The general discussion in chapter 8 integrates findings across this thesis, placing them in context of ongoing research, and offering directions for future studies and clinical implementation.

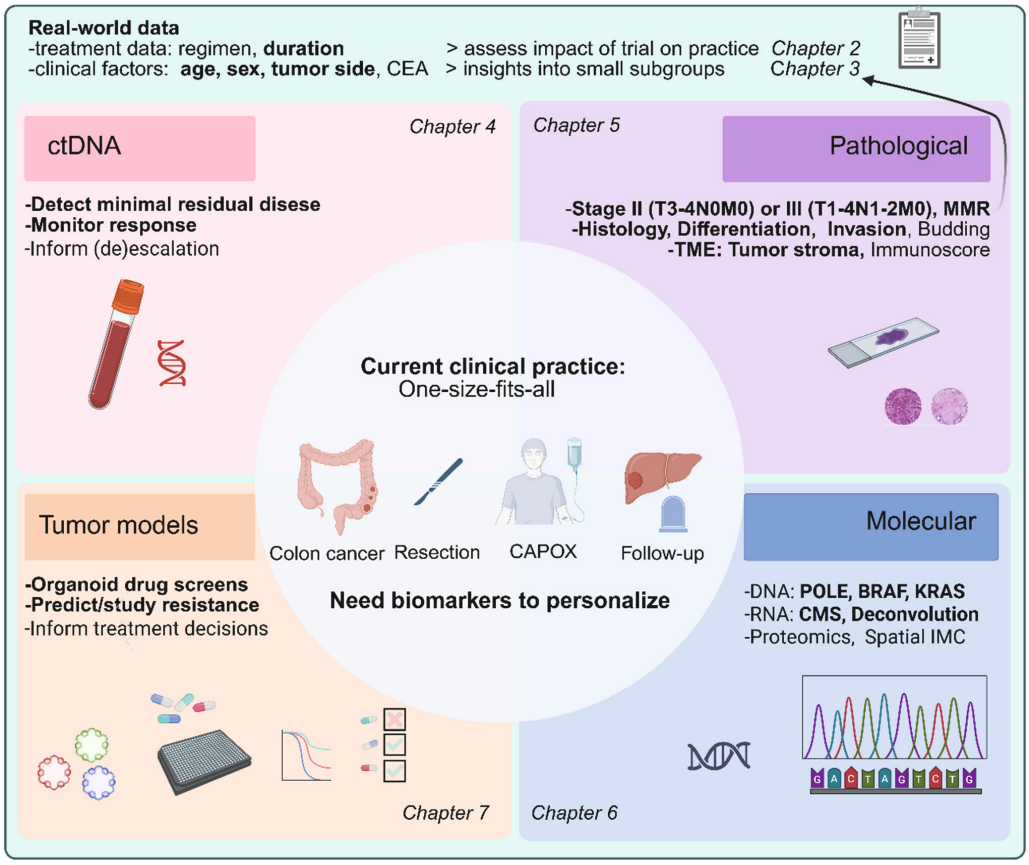

Figure 1: Overview of real-world data and biomarkers in stage II/III colon cancer, to improve prognostication after adjuvant CAPOX and to inform personalized treatment. In bold are the biomarkers investigated in the indicated chapters in this thesis.
CAPOX, capecitabine and oxaliplatin; CEA, carcinoembryonic antigen; CMS, consensus molecular subtype; ctDNA, circulating tumor DNA; IMC, imaging mass spectrometry; MMR, mismatch repair; TME; tumor microenvironment.

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Part I

Nationwide registry data to describe clinical practice in the general CRC population

2

Survival and patient-reported outcomes of real-world high-risk stage II and stage III colon cancer patients after reduction of adjuvant CAPOX duration from 6 to 3 months

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Abstract

Introduction

Adjuvant chemotherapy has been advised for high-risk stage II and III colon cancer since 2004. After the IDEA study showed no clinically relevant difference in outcome, reduction of adjuvant CAPOX duration from 6 to 3 months was rapidly adopted in the Dutch treatment guideline in 2017. This study investigates the real-world impact of the guideline change on overall survival (OS) and patient-reported outcomes (PROs).

Methods

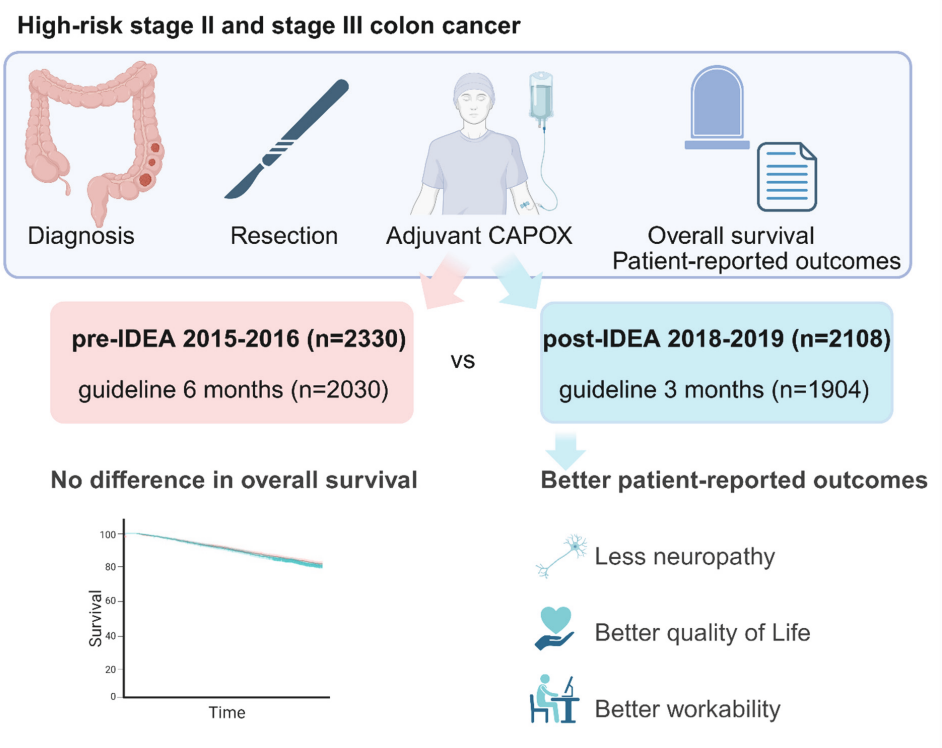
Patients with high-risk stage II (pT4 +) and III (pN+) colon cancer were selected from the Netherlands Cancer Registry, based on surgical resection and adjuvant CAPOX before (2015–2016) versus after (2018–2019) the guideline change. Both groups were compared based on OS, using multivariable Cox regression, and on PROs.

Results

Patients treated before (n=2330) and after (n=2108) the guideline change showed similar OS (HR 1.02 [95%CI 0.89–1.16]), also in high-risk stage III (pT4/N2, HR 1.06 [0.89–1.26]). After the guideline change, 90% of patients were treated for 3 months with no inferior OS to those still receiving 6 months (HR 0.89 [0.66–1.20]). PROs 2 years after CAPOX completion, available for a subset of patients, suggest a lower neuropathy (n=366; 26.2 [21.3–31.1] to 16.5 [14.4–18.6]) and better quality of life (n=396; 80.9 [78.6–83.2] to 83.9 [82.8–84.9]), but no significant difference in workability (n=120; 31.5 [27.9–35.1]) to 35.3 [33.8–36.7]), with reduction from 6 to 3 months of CAPOX.

Conclusion

This real-world study confirmed that shorter adjuvant CAPOX did not compromise OS and may improve PROs, complementing the IDEA study and supporting 3 months of adjuvant CAPOX in daily clinical practice.


High-risk stage II and stage III colon cancer
Diagnosis
Resection
Adjuvant CAPOX
Overall survival
Patient-reported outcomes
pre-IDEA 2015-2016 (n=2330)
guideline 6 months (n=2030)
vs
post-IDEA 2018-2019 (n=2108)
guideline 3 months (n=1904)
No difference in overall survival
Survival
100
80
60
40
20
0
Time
Better patient-reported outcomes
Less neuropathy
Better quality of Life
Better workability

Introduction

Patients with colon cancer classified as high-risk stage II (pT4 or other risk factors) or stage III (pN+ involvement of local lymph nodes) are at increased risk of disease recurrence.[1] Compared to surgical resection only, systemic adjuvant chemotherapy (ACT) with a fluoropyrimidine (5FU or capecitabine) improved prognosis[1,2], with additional benefit from oxaliplatin (FOLFOX or CAPOX, respectively).[3,4] This doublet has been standard of care since 2004[5], although at the expense of oxaliplatin-associated sensory peripheral neuropathy[3,6] and lower workability[7].

The international duration evaluation of adjuvant chemotherapy (IDEA) study found that the reduction of 6 months to 3 months doublet ACT was able to reduce neuropathy[8,9], improve quality of life, and lower costs[10,11]. Moreover, this shorter treatment duration was non-inferior for CAPOX, but non-inferiority was not met for the high-risk stage III subgroup and for FOLFOX.[8,12] Based on these results, international guidelines adhere to 6 months of CAPOX or FOLFOX in high-risk stage III (pT4 and/or pN2), while recommending 6 months of FOLFOX or 3 months of CAPOX in low-risk stage III (pT1–3N1). In stage II colon cancer (pT3–4N0), guidelines differ in risk factors to consider whether benefit outweighs harm of ACT, using fluoropyrimidine monotherapy (6 months) or also oxaliplatin (6 or 3 months).[13,14]

The Dutch guideline changed in 2017 from 6 to 3 months of CAPOX for both stage III and high-risk stage II, redefined as only pT4 combined with proficient mismatch repair status (pMMR).[5] A previous publication using Dutch population-based data between 2015 and 2019 demonstrated quick implementation of the guideline in clinical practice, with a reduction of mean ACT duration from 18.6 to 9.5 weeks. With the shorter duration, more patients received ACT (stage III 61% to 69%), with a larger proportion of doublet ACT (74% to 83% CAPOX).[15] This quick and broad implementation of the guideline change offers the unique opportunity to investigate its effect in daily clinical practice, resembling ‘random’ treatment assignment in a large natural experiment.

Our study investigates the generalizability of the IDEA results to a real-world population of patients with high-risk stage II and II colon cancer, hypothesizing that the reduced adjuvant CAPOX duration from 6 to 3 months is not associated with an inferior overall survival, while benefitting patient-reported neurotoxicity, quality of life and workability.

Methods

Study population

As depicted in the flowchart (Figure 1), all adult patients diagnosed with pathologically confirmed high-risk stage II (pT4) and stage III (pN+) colon cancer in 2015-2019 and treated with surgical resection and adjuvant CAPOX without neoadjuvant treatment were selected from the Netherlands Cancer Registry (NCR). NCR is hosted by the Netherlands Comprehensive Cancer Organisation (IKNL) and has near complete coverage (>99%) of all newly diagnosed malignancies.[16] Adjuvant CAPOX, the ACT regimen used in circa 80% in the Netherlands[15], was defined as capecitabine with at least one simultaneous cycle of oxaliplatin. Patients treated in the guideline transition year 2017 or with an unknown number of cycles of CAPOX were excluded because of unknown treatment duration. Also patients surviving <3 months after CAPOX initiation were excluded to avoid immortal time bias between 3 and 6 months of CAPOX.

Study variables

Information on patient and tumor characteristics and treatment was routinely collected from medical records by trained administrators in the year of diagnosis. Key variables such as year, number of CAPOX cycles, pathological stage, age, and sex were complete. Other baseline variables include American Society of Anesthesiologists (ASA) physical status, tumor sidedness (proximal versus distal to splenic flexure), prior malignancy, radicality of resection, differentiation grade, number of lymph nodes dissected, lymphatic invasion (all <10 % missing), vascular invasion (17 % missing), and MMR status (34 % missing). Missing data was accounted for by multiple regression-based imputation on all mentioned baseline variables and the auxiliary variable world health organization (WHO) performance status. Overall survival (OS) was derived from the national municipal population registry in January 2024 and calculated from start of adjuvant CAPOX and was censored at loss to follow-up.

Approaches to classify CAPOX duration

Three complementary approaches were used to classify CAPOX duration for association with OS, all excluding 2017 as guideline transition year (Figure 1). First, to evaluate the impact of the guideline change, OS was compared between patients treated with adjuvant CAPOX before (2015–2016) versus after (2018–2019) the guideline change. Second, to assess the robustness of the results after the guideline

change (2018–2019), a post-IDEA sensitivity analysis was performed between patients still receiving 5–8 cycles versus patients adhering to the changed guideline with 1–4 cycles. Third, patients were compared between an intended duration of 6 months (all patients treated in 2015–2016 and patients still receiving 5–8 cycles in 2018–2019) and intended duration of 3 months (1–4 cycles in 2018–2019).

Overall survival

For all three approaches, OS was assessed in Kaplan Meier curves with 5-year survival rates as main outcome (81 % complete follow-up) and 3-year survival rates supplementary (>99 % complete). Hazard ratios (HRs) with 95 % confidence intervals (CIs) were generated using both univariable log-rank analysis and a multivariable Cox regression model including the 12 preselected baseline factors. All OS analyses were performed in the overall population and in prespecified subgroup analyses focusing on high-risk stage III, while pooling low-risk stage III and high-risk stage II. Interactions between CAPOX duration and other variables were assessed. Variables that visibly and statistically based on Schoenfeld residuals did not meet the proportional hazards assumption were modelled as stratification factors.

PROs

For a subset of patients, longitudinal patient-reported outcomes (PROs) were available through the Prospective Dutch Colorectal Cancer (PLCRC) cohort (NCT02070146).[17] To assess the influence of reduction of CAPOX duration on long-term PROs, questionnaires were selected closest to two years after treatment cessation (min one–max three years). Chemotherapy-induced polyneuropathy (CIPN20) was based on 9 sensory, 8 motor and 3 autonomic symptoms over the past week.[18] With the exception of driving a car and erection due to their conditional nature[19], all items were combined to (sub)total scores and linearly transformed to a scale from 0 (‘not at all’) to 100 (‘very much’). The EORTC-QLQ-C30′s 9 cancer-related symptoms (0–100 ‘no-maximal’) and 5 functional scales (physical, role, emotional, cognitive and social functioning; 0–100 ‘worst-best’) were used to calculate the quality of life (QOL) summary score.[20,21] For patients aged <67 years with a paid job, the current workability compared to best ever (rated 1–10), number of comorbidities, work impairment, sick leave, expected workability in next two years and vitality were used to determine the workability index (WAI score 7–27 ‘poor’, 28–36 ‘moderate’, 37–43 ‘good’, 44–49 ’excellent’).[7,22]

Statistical methods

After confirming similar baseline characteristics, PROs were compared between patients with an intended CAPOX duration of 6 versus 3 months, using the Mann-Whitney U test for continuous scores (mean and standard deviation (SD)) and χ^2 test for categorical items. All statistical tests were performed using R (v3.5.1), with a two-sided p-value of <0.05 considered statistically significant, without correction for multiple testing. R packages include *table1*, *mice*, *ggplot2*, *survival*, *survminer* and *ggsurvfit*.

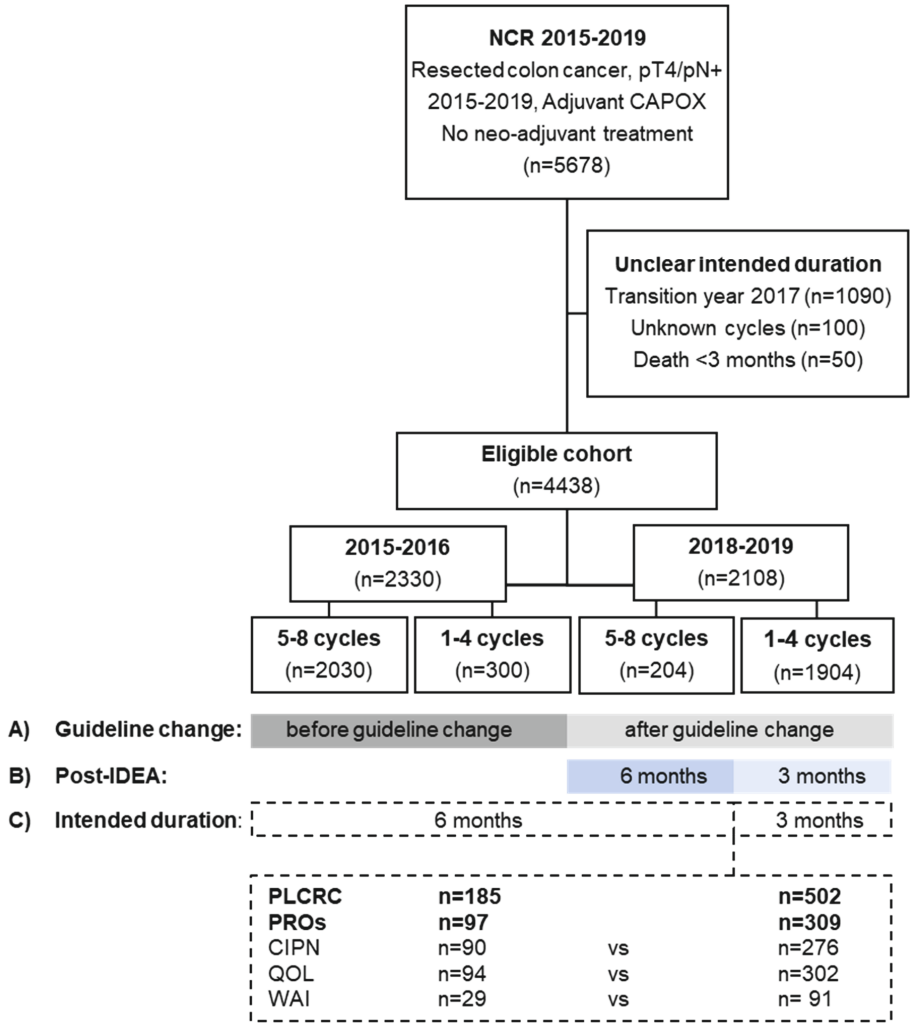


Figure 1: Flow diagram of the study population, and the three approaches of analyzing CAPOX duration.
CIPN, chemotherapy-induced polyneuropathy; NCR, Netherlands Cancer Registry; PLCRC, Prospective Dutch Colorectal Cancer cohort; PROs, patient-reported outcomes; QOL, quality of life; WAI workability index.

Results

Study population

Of the 5678 colon cancer patients identified in the NCR with adjuvant CAPOX in 2015–2019, 4438 were eligible after excluding the guideline transition year 2017 (19%), an unclear number of CAPOX cycles (2%) or <3 months survival (1%) (Figure 1). Of the 2330 patients treated before the guideline change (2015–2016), 2030 (87%) completed 5–8 cycles conform the recommended duration. The other 2108 patients received adjuvant CAPOX after the guideline change (2018–2019), of whom 204 (10%) still received 5–8 cycles, while 1904 (90 %) received 1–4 cycles in accordance with the changed guideline.

The overall study population's mean age was 63 years (SD 8.6), 55 % was male, 87 % ASA1 or ASA2, 56 % harbored a left-sided tumor and 90 % experienced no prior malignancy. Based on pathological TNM, 6% was high-risk stage II (pT4N0), 53 % low-risk stage III (pT1–3N1) and 41 % high-risk stage III (pT4/N2). The majority of patients had a R0 resection (98%), good or moderate differentiation (88%), ≥0 lymph nodes dissected (96%), no lymphatic invasion (66%), no vascular invasion (72%) and proficient MMR status (86%). These baseline characteristics were comparable between patients treated before versus after the guideline change (Table 1; more detailed in Supplementary Table 1).

Guideline change: before (2015-2016) versus after (2018-2019) the guideline change

Patients treated with adjuvant CAPOX before (n=2330 in 2015–2016) and after (n=2108 in 2018–2019) the guideline change had a median follow-up of 91 months (IQR 8 months) and 57 months (IQR 7 months), respectively. After both 3 years (Supplementary Table 2) and 5 years (Table 2), survival was not significantly different between patients treated before (5-year OS 81%; [95%CI 79–82]) and after the guideline change (5-year OS 80 % [78–81], HR 1.02 [0.89–1.16] based on the multivariable Cox model in Supplementary Table 3) (Figure 2A). Also the subgroup with high-risk stage III showed similar OS before (n=992, 5-year OS 71% [68–74]) and after (n=824, 5-year OS 68% [65–72]) the guideline change (HR 1.06 [0.89–1.26]) (Table 2).

Table 1: Baseline characteristics before (2015-2016, recommended duration 6 months) versus after (2018-2019, recommended duration 3 months) the guideline change.
ASA, American society of anesthesiologists; TNM, tumor-node-metastasis.

	Before guideline change (2015-2016) 6 months CAPOX n=2330	**After guideline change (2018-2019)** 3 months CAPOX n=2108	**Overall** n=4438
Pathological TNM stage			
High-risk stage II	133 (5.7%)	139 (6.6%)	272 (6.1%)
Low-risk stage III	992 (42.6%)	824 (39.1%)	2350 (53.0%)
High-risk stage III	1205 (51.7%)	1145 (54.3%)	1816 (40.9%)
Age			
Mean (SD)	62.9 (8.5)	63.0 (9.7)	62.9 (9.1)
Sex			
Male	1321 (56.7%)	1101 (52.2%)	2422 (54.6%)
Female	1009 (43.3%)	1007 (47.8%)	2016 (45.4%)
ASA physical status			
ASA1	582 (27.0%)	382 (18.8%)	964 (23.0%)
ASA2	1347 (62.4%)	1280 (63.1%)	2627 (62.8%)
ASA3-4	228 (10.6%)	366 (18.0%)	594 (14.2%)
Missing	173	80	253
Tumor sidedness			
Left	1324 (57.4%)	1141 (54.7%)	2465 (56.1%)
Right	984 (42.6%)	946 (45.3%)	1930 (43.9%)
Missing	22	21	43
Prior malignancy			
No	2117 (90.9%)	1873 (88.9%)	3990 (89.9%)
Yes	213 (9.1%)	235 (11.1%)	448 (10.1%)
Radical resection			
R0	2256 (98.0%)	2035 (98.3%)	4291 (98.1%)
R1 or R2	46 (2.0%)	35 (1.7%)	81 (1.9%)
Missing	22	38	66
Differentiation grade			
Good-moderate	1860 (87.4%)	1751 (88.6%)	3611 (88.0%)
Poor-undifferentiated	268 (12.6%)	226 (11.4%)	494 (12.0%)
Missing	202	131	333
Lymph nodes			
≥10 dissected	2242 (96.2%)	2027 (96.2%)	4269 (96.2%)
<10 dissected	88 (3.8%)	81 (3.8%)	169 (3.8%)
Lymphatic invasion			
No invasion	1530 (68.0%)	309 (63.4%)	2839 (65.8%)
Lymphatic invasion	720 (32.0%)	756 (36.6%)	1476 (34.2%)
Missing	80	43	123
Vascular invasion			
No invasion	1141 (71.3%)	1487 (71.9%)	2628 (71.6%)
Vascular Invasion	460 (28.7%)	580 (28.1%)	1040 (28.4%)
Missing	729	41	770
Mismatch repair status			
Proficient	1010 (87.1%)	1499 (85.9%)	2509 (86.4%)
Deficient	150 (12.9%)	246 (14.1%)	396 (13.6%)
Missing	1170	363	1533

Post-IDEA: 6 versus 3 months CAPOX after the guideline change (2018-2019)

After the guideline change in 2017, patients could receive the recommended 3 months (n=1904) or still 6 months of CAPOX (n=204, 10%) based on shared decision making. Patients still receiving 6 months tended to be younger (61 versus 63 years), with more stage T4 and/or N2 tumors (67% versus 26%) and poor pathological characteristics (Supplementary Table 1). Compared with survival after 6 months of CAPOX (5-year OS 73% [67–79]), survival after 3 months of CAPOX (5-year OS 80% [79–82]) was better in univariable analysis (HR 0.68 [0.51–0.91]), but not when correcting for the baseline differences (HR 0.89 [0.66–1.20]) or when stratifying for high-risk stage III (HR 0.87 [0.63–1.19]) (Figure 2B, Table 2).

Intended duration: 6 months (2015-2019) versus 3 months (2018-2019)

Lastly, patients still receiving 5–8 cycles after the guideline change (n=204) were combined with all patients treated before the guideline change (n=2330) as group with intended CAPOX duration of 6 months. In comparison, patients receiving 1–4 cycles after the guideline change (n=1904) with an intended duration of 3 months did not show clinically relevant differences in baseline characteristics (Supplementary Table 1) or survival (both 5-year OS 80% [79–82], HR 0.99 [0.86–1.13]) (Figure 2C, Table 2).

Table 2: 5-year survival after 6 months (ref) versus 3 months of CAPOX, according to three approaches: A) Guideline change: before (2015-2016) versus after (2018-2019) (2015-2016 versus 2018-2019); B) Post-IDEA: 6 (2018-2019) versus 3 months (2018-2019); C) Intended duration: 6 (2015-2019) versus 3 months (2018-2019).
CI, confidence interval [lower–upper bound]; HR, hazard ratio; OS, overall survival.

	6 months CAPOX			3 months CAPOX			Univariable		Multivariable	
	n	OS%	95%CI	n	OS%	95%CI	HR	95%CI	HR	95%CI
A) Guideline change										
all patients	2330	81%	[79-82]	2108	80%	[78-81]	1.07	[0.93-1.22]	1.02	[0.89-1.16]
high-risk stage III	992	71%	[68-74]	824	68%	[65-71]	1.14	[0.96-1.36]	1.06	[0.89-1.26]
low-risk stage III/II	1338	88%	[86-90]	1284	87%	[85-89]	1.06	[0.85-1.32]	0.95	[0.76-1.19]
B) Post-IDEA										
all patients	204	73%	[67-80]	1904	80%	[79-82]	0.68	[0.51-0.91]	0.89	[0.66-1.20]
high-risk stage III	136	65%	[58-74]	688	69%	[65-72]	0.86	[0.63-1.18]	0.87	[0.63-1.19]
low-risk stage III/II	68	89%	[81-97]	1216	87%	[85-89]	1.21	[0.57-2.58]	1.23	[0.57-2.66]
C) Intended duration										
all patients	2534	80%	[79-82]	1904	80%	[79-82]	0.98	[0.86-1.13]	0.99	[0.86-1.13]
high-risk stage III	1128	70%	68-73]	688	69%	[65-72]	1.08	[0.90-1.28]	1.01	[0.85-1.21]
low-risk stage III/II	1406	88%	[86-90]	1216	87%	[85-89]	1.08	[0.86-1.34]	0.98	[0.78-1.23]

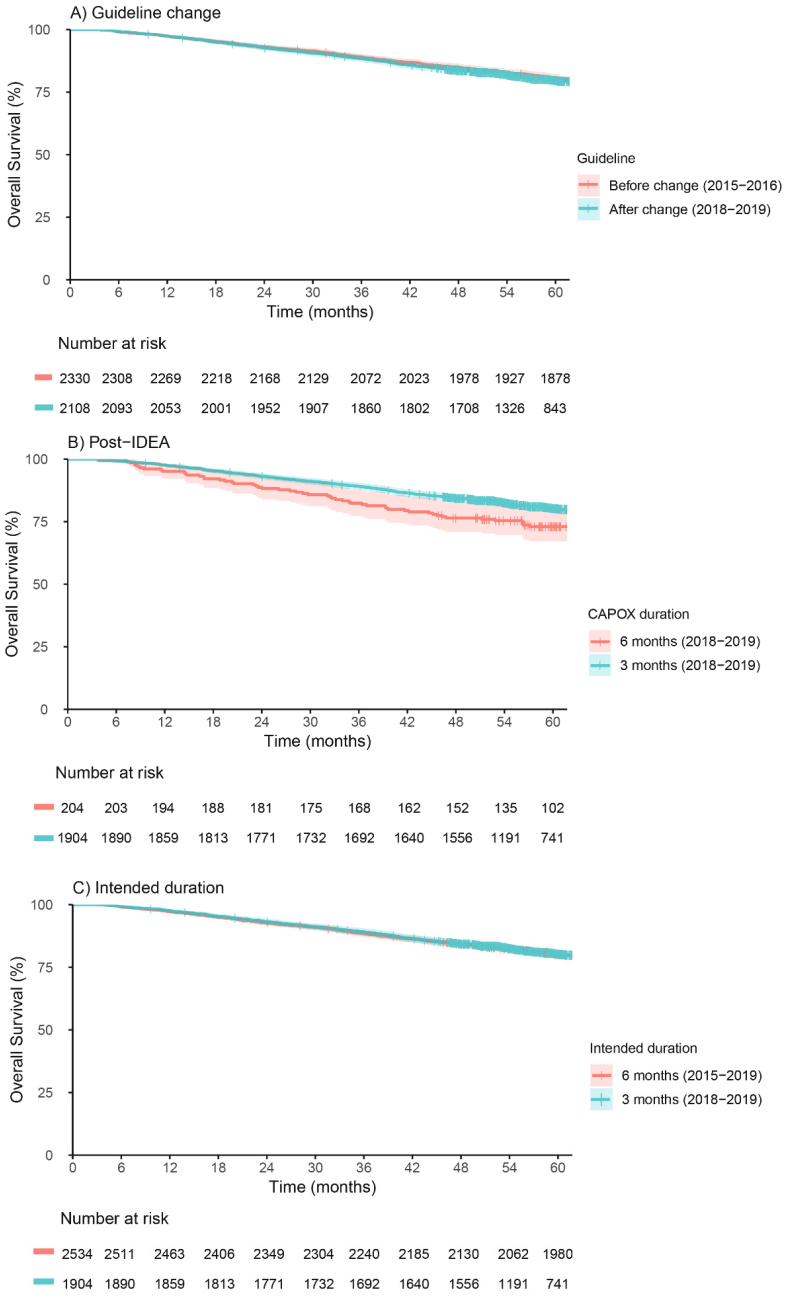


Figure 2: Kaplan Meier of overall survival based on the three approaches of analyzing CAPOX duration. A) Guideline change: before (2015-2016) versus after (2018-2019) (2015-2016 versus 2018-2019); B) Post-IDEA: 6 (2018-2019) versus 3 months (2018-2019); C) Intended duration: 6 (2015-2019) versus 3 months (2018-2019).

Effect of 6 months (2015-2019) versus 3 months (2018-2019) CAPOX on PROs

Of patients with an intended duration of 6 and 3 months, respectively 7 % and 26 % participated in PLCRC, through which 4% and 16% had available prospective questionnaires two years after ACT. Baseline characteristics were comparable between the subgroup with PROs and the total cohort with OS data, as well as between patients with an intended duration of 6 and 3 months (Supplementary Table 4). Patient reports (Supplementary Table 5-7) were used to generate summary scores (Table 3). Compared with an intended duration of 6 months of CAPOX (n=90, mean 26.2 [21.3–31.1]), the CIPN total score was lower after 3 months of CAPOX (n=276, mean 16.5 [14.4–18.6]) (p<0.001), with the largest difference in the sensory subscore (especially tingling, burning pain or numbness in hands or feet) (Supplementary Table 5).

The quality of life summary score two years after CAPOX improved with reduction from 6 months (n=94, mean 80.9 [78.6–83.2]) to 3 months (n=302, mean 83.9 [82.8–84.9]) (p=0.013, Table 3). Especially role functioning, social functioning and cancer related symptoms (Supplementary Table 6). Among the small group of patients with income from work, the WAI score did not differ significantly between 6 months (n=29, mean 31.5 [27.9–35.1]) versus 3 months (n=91, mean 35.3 [33.8–36.7], p=0.12), except for self-assessed current workability (6.6 [5.6–7.7] versus 7.6 [7.2–8.1], with 10 ‘best ever’, p=0.042) (Supplementary Table 7).

Table 3 PROs two years after completion of 6 months versus 3 months of intended CAPOX duration.
CI, confidence interval; CIPN, chemotherapy-induced neuropathy; M, mean; n, number of patients with available PROs; QoL, quality of life; WAI, workability index.

	6 months CAPOX			3 months CAPOX			Difference	
	n	**M**	**95%CI**	**n**	**M**	**95%CI**	**M**	**95%CI**
CIPN total score (0-100)	90	26.2	[21.3-31.1]	276	16.5	[14.4-18.6]	-9.7	[-15.0 - 4.4]
Sensory sub score (0-100)		27.5	[22.4-32.7]		17.3	[15.0-19.7]	-10.2	[-15.8 - -4.6]
Motor sub score (0-100)		17.6	[13.4-21.8]		11.4	[9.6-13.2]	-6.2	[-10.9 - -1.9]
Autonomic sub score (0-100)		14.4	[9.6-19.3]		8.8	[7.0-10.6]	-5.6	[-10.7 - -0.5]
QoL summary score (0-100)	94	80.9	[78.6-83.2]	302	83.9	[82.8-84.9]	+3.0	[0.5 - 5.5]
Global health status (0-100)		73.3	[69.2-77.5]		79.1	[77.2-81.1]	+7.8	[1.2 - 10.3]
WAI score (7-49)	29	31.5	[27.9-35.1]	91	35.3	[33.8-36.7]	+3.8	[-0.6 - 7.6]
Compared to best ever (1-10)		6.6	[4.6-7.7]		7.6	[7.2-8.1]	+1.0	[-0.1 - 2.0]

Discussion

This large real-world population-based cohort of patients with high-risk stage II and III colon cancer confirmed that the IDEA-based guideline change from 6 to 3 months of adjuvant CAPOX was not associated with inferior OS. This was confirmed in a sensitivity analysis, comparing patients with 6 versus 3 months post-IDEA and in TNM-based subgroup analyses. The high-risk stage III subgroup was of special interest, because of the international ongoing debate after the IDEA study failed to show non-inferiority (5-year OS 72.4% to 71.4%, HR 1.03 [0.89–1.02]).[23,24] In the Netherlands, 3 months was adopted also for high-risk stage III[15,25], allowing us to be the first to robustly show that 3 months of CAPOX was not associated with inferior OS in the real-world population.

Another core finding supporting shorter CAPOX duration is the improved long-term patient-reported outcome. First, we confirmed the lower CIPN following reduced doublet ACT duration observed in the IDEA study.[26] Additionally, we found that shorter CAPOX duration was associated with better quality of life, especially role and social functioning, even after two years when recovery to baseline is expected.[27] Lastly, workability was regained slightly better in some subscales. This should be interpreted cautiously, considering the limited number of available PROs. However, we expect participation bias[28] to be limited because of the comparable baseline characteristics.

The main limitation of this observational study is the vulnerability to potential bias, which was mitigated through various analysis steps. First, the guideline change resembled ‘random’ treatment assignment between two groups with otherwise comparable characteristics, assumably rendering CAPOX duration the only varying independent variable and limiting confounding by indication.[29] To address the confounding characteristics of the 10% of patients still receiving 5–8 cycles post-IDEA, namely advanced tumor stage (T4 and/or N2) potentially resulting in an underestimation, a separate sensitivity analysis was performed. Multivariable analyses adjusted for known relevant confounders (including stage, age and sex)[30], although residual confounding cannot be ruled out.[31,32]

Core variables contained no missing values and were determined before patients started CAPOX, hence restricting post-baseline confounding.[33] The chosen endpoint 5-year OS is the optimal endpoint in trials investigating ACT aimed at curation. It should be noted that the immaturity of 5-year OS for patients treated in 2019 may cause an underestimation of the effect of the guideline change, although the trend observed in the Kaplan Meier was constant and consistent with mature 3-year OS. Disease-free survival was not available and would be of interest in the future, although 3-year DFS has been shown to correlate with 5-year OS.[34]

The main strength of this study is the population-wide sample size, minimizing selection bias and optimizing generalizability. Since randomized controlled trials often select young patients with less comorbidities, treatment benefit and toxicity may be inferior when applied to a broader population.[35,36] This gap between trial efficacy and real-world effectiveness has been reported in metastatic colorectal cancer[37], but was limited in real-world data concerning the addition of ACT to surgery in stage II[38] or stage III[39] colon cancer. Also in our population-based ACT cohort, patient characteristics and survival were comparable to the IDEA study (5-year OS 81.2 % to 82.1 %, HR 0.96 [0.85–1.08])[8,12], likely thanks to the trial's broad selection criteria. These findings support pragmatic trial designs that better approximate clinical practice and translate to real-world effectiveness.[29,40]

In conclusion, reduced adjuvant CAPOX duration from 6 to 3 months was not associated with worse OS, also in high-risk stage III. Importantly, long-term PROs in a small subset of patients suggested less neurotoxicity and higher quality of life. This study thereby confirms and complements the findings of the pivotal IDEA study in a real-world population, supporting 3 months of adjuvant CAPOX in daily clinical practice.

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Supplementary material

Supplementary Table 1: Baseline characteristics of patients receiving 6 versus 3 months of CAPOX, before versus after the guideline change.
ASA, American society of anesthesiologists; SD, standard deviation; TNM, tumor-node-metastasis.

	Intended 6 months			Intended 3 months
	2015-2016 5-8 cycles n=2030	2015-2016 1-4 cycles n=300	2018-2019 5-8 cycles n=204	2018-2019 1-4 cycles n=1904
Pathological TNM stage				
High-risk stage II	100 (4.9%)	33 (11.0%)	20 (9.8%)	119 (6.3%)
Low-risk stage III	1038 (51.1%)	167 (55.7%)	48 (23.5%)	1097 (57.6%)
High-risk stage III	892 (43.9%)	100 (33.3%)	136 (66.7%)	688 (36.1%)
Age Mean (SD)	62.7 (8.52)	64.8 (7.74)	60.7 (9.92)	63.2 (9.62)
Sex				
Male	1183 (58.3%)	138 (46.0%)	107 (52.5%)	994 (52.2%)
Female	847 (41.7%)	162 (54.0%)	97 (47.5%)	910 (47.8%)
ASA physical status				
ASA1	519 (27.5%)	63 (23.1%)	46 (23.5%)	336 (18.3%)
ASA2	1176 (62.4%)	171 (62.6%)	126 (64.3%)	1154 (63.0%)
ASA3-4	189 (10.0%)	39 (14.3%)	24 (12.2%)	342 (18.7%)
Missing	146	27	8	72
Tumor sidedness				
Left	1180 (58.7%)	144 (48.3%)	105 (51.7%)	1036 (55.0%)
Right	830 (41.3%)	154 (51.7%)	98 (48.3%)	848 (45.0%)
Missing	20	2	1	20
Prior malignancy				
No	1851 (91.2%)	266 (88.7%)	187 (91.7%)	1686 (88.6%)
Yes	179 (8.8%)	34 (11.3%)	17 (8.3%)	218 (11.4%)
Radical resection				
R0	1965 (98.0%)	291 (98.3%)	191 (96.0%)	1844 (98.6%)
R1 or R2	41 (2.0%)	5 (1.7%)	8 (4.0%)	27 (1.4%)
Missing	24	4	5	33
Differentiation grade				
Good-moderate	1622 (87.4%)	238 (87.2%)	153 (82.7%)	1598 (89.2%)
Poor-undifferentiated	233 (12.6%)	35 (12.8%)	32 (17.3%)	194 (10.8%)
Missing	175	27	19	112
Lymph nodes				
≥10 dissected	1949 (96.0%)	293 (97.7%)	195 (95.6%)	1832 (96.2%)
<10 dissected	81 (4.0%)	7 (2.3%)	9 (4.4%)	72 (3.8%)
Lymphatic invasion				
No invasion	1331 (67.8%)	199 (69.3%)	113 (56.8%)	1196 (64.1%)
Lymphatic invasion	632 (32.2%)	88 (30.7%)	86 (43.2%)	670 (35.9%)
Missing	67	13	5	38
Vascular invasion				
No invasion	1004 (71.3%)	137 (71.0%)	131 (65.8%)	1356 (72.6%)
Vascular Invasion	404 (28.7%)	56 (29.0%)	68 (34.2%)	512 (27.4%)
Missing	622	107	5	36
Mismatch repair status				
Proficient	883 (88.0%)	127 (80.9%)	150 (89.3%)	1349 (85.5%)
Deficient	120 (12.0%)	30 (19.1%)	18 (10.7%)	228 (14.5%)
Missing	1027	143	36	327

Supplementary Table 2: 3-year overall survival after 6 months (ref) versus 3 months of CAPOX, according to three approaches: A) Guideline change: before (2015-2016) versus after (2018-2019) (2015-2016 versus 2018-2019); B) Post-IDEA: 6 (2018-2019) versus 3 months (2018-2019); C) Intended duration: 6 (2015-2019) versus 3 months (2018-2019). Multivariable HR conform Cox models in Supplementary Table 3.
CI, 95% confidence interval [lower–upper bound]; HR, hazard ratio; OS, overall survival after 3 years (%).

	6 months CAPOX			3 months CAPOX			Univariable		Multivariable	
	n	OS	CI	n	OS	CI	HR	CI	HR	CI
A) Guideline change										
all patients	2330	89%	[88-90]	2108	89%	[87-90]	1.05	[0.88-1.25]	0.99	[0.83-1.19]
high-risk stage III	992	82%	[80-84]	824	80%	[77-83]	1.12	[0.91-1.39]	1.04	[0.84-1.29]
low-risk stage III/II	1338	94%	[93-96]	1284	94%	[93-95]	1.05	[0.76-1.43]	0.95	[0.65-1.25]
B) Post-IDEA										
all patients	204	82%	[77-88]	1904	89%	[88-91]	0.59	[0.41-0.84]	0.78	[0.54-1.12]
high-risk stage III	136	77%	[70-84]	688	81%	[78-84]	0.80	[0.54-1.18]	0.87	[0.63-1.18]
low-risk stage III/II	68	94%	[89-100]	1216	94%	[93-95]	1.03	[0.38-2.82]	1.15	[0.42-3.20]
C) Intended duration										
all patients	2534	89%	[87-90]	1904	89%	[88-91]	0.94	[0.78-1.12]	0.94	[0.78-1.13]
high-risk stage III	1128	81%	[79-84]	688	81%	[78-84]	1.04	[0.83-1.29]	0.98	[0.78-1.13]
low-risk stage III/II	1406	94%	[93-96]	1216	94%	[93-95]	1.05	[0.76-1.43]	0.92	[0.67-1.27]

Supplementary Table 3: Hazard ratios per factor in the multivariable Cox models for 5-year overall survival, according to the three approaches: A) Guideline change: before (2015-2016) versus after (2018-2019) (2015-2016 versus 2018-2019); B) Post-IDEA: 6 (2018-2019) versus 3 months (2018-2019); C) Intended duration: 6 (2015-2019) versus 3 months (2018-2019).
* internal stratification because of violated proportional hazard assumption.
ASA, American society of anesthesiologists; CI, 95% confidence interval [lower–upper bound]; HR, hazard ratio; st, stage.

			A) Guideline change			B) Post-IDEA			C) Intended duration		
			All patients	High-risk st III	Low-risk st III +high-risk st II	All patients	High-risk st III	Low-risk st III +high-risk st II	All patients	High-risk st III	Low-risk st III +high-risk st II
Category	reference	level	**HR [95%CI]**	**HR [95%CI]**	**HR [95%CI]**	**HR [95%CI]**	**HR [95%CI]**	**HR [95%CI]**	**HR [95%CI]**	**HR [95%CI]**	**HR [95%CI]**
Duration	6 months	3months	1.02 [0.89−1.16]	1.06 [0.89−1.26]	0.95 [0.76−1.19]	0.89 [0.66-1.20]	0.87 [0.63-1.19]	1.23 [0.57-2.66]	0.99 [0.86-1.13]	1.01 [0.85-1.21]	0.98 [0.78-1.22]
Stage	High-rsk st II	Low-risk st III	0.75 [0.54−1.03]	NA	[0.53−1.03]	0.70 [0.44-1.09]	NA	0.70 [0.44-1.12]	0.75 [0.54-1.03]	NA	0.75 [0.53-1.04]
		High-risk st III	1.88 [1.37−2.58]	NA	NA	1.91 [1.23-2.95]	NA	NA	1.87 [1.36-2.58]	NA	NA
Age			1.01 [1.00−1.02]	1.01 [0.99−1.02]	1.02 [1.01−1.04]	1.02 [1.01-1.03]	1.02 [1.01-1.04]	1.02 [1.00-1.04]	1.01 [1.00-1.02]	1.01 [0.99-1.02]	1.02 [1.01-1.04]
Sex	Male	Female	1.00 [0.88−1.15]	1.03 [0.86−1.22]	0.95 [0.76−1.20]	1.01 [0.83-1.23]	1.03 [0.81-1.31]	0.98 [0.71-1.37]	1.01 [0.88-1.15]	1.03 [0.86-1.22]	0.95 [0.76-1.20]
ASA	ASA1	ASA2	1.46 [1.19−1.77]	1.50 [1.17-1.92]	1.41 [1.01-1.97]	1.22 [0.89-1.66]	1.20 [0.82-1.74]	1.29 [0.76-2.21]	1.46 [1.20-1.78]	*	1.41 [1.01-1.96]
		ASA3-4	2.26 [1.78−2.86]	2.25 [1.67-3.04]	2.36 [1.59-3.52]	1.79 [1.26-2.54]	1.55 [1.00-2.40]	2.42 [1.34-4.35]	2.27 [1.79-2.88]	*	2.35 [1.58-3.50]
Tumor sidedness		Right	*	1.50 [1.25-1.81]	1.34 [1.05-1.72]	1.53 [1.23-1.89]	[1.20-2.04]	1.40 [0.99-1.98]	1.40 [0.99-1.98]	*	1.34 [1.05-1.72]
Prior malignancy	No	Yes	1.23 [1.01−1.51]	0.96 [0.73−1.27]	1.75 [1.30−2.37]	1.43 [1.10-1.87]	1.12 [0.78-1.59]	2.28 [1.54-3.39]	2.28 [1.54-3.39]	0.96 [0.73-1.26]	1.75 [1.30-2.37]
Radical resection	R0	R1 or R2	1.46 [0.99−2.14]	1.29 [0.85−1.97]	2.74 [1.12−6.75]	1.83 [1.11-3.02]	1.67 [0.98-2.84]	4.20 [0.96-18.4]	4.20 [0.99-2.14]	1.30 [0.85-1.98]	2.75 [1.12-6.78]
Differentiation	Good-moderate	Poor-undiff	*	1.37 [1.08-1.73]	1.49 [1.02-2.18]	1.20 [0.89-1.62]	*	1.25 [0.70-2.25]	*	1.38 [1.08-1.75]	1.49 [1.02-2.18]
Lymph nodes dissected	≥10	<10	1.15 [0.80−1.66]	1.11 [0.64−1.94]	1.11 [0.68−1.80]	1.36 [0.85-2.20]	1.54 [0.78-3.02]	1.08 [0.54-2.15]	1.15 [0.80-1.65]	1.13 [0.65-1.98]	1.10 [0.68-1.80]
Lymphatic invasion	No	Yes	1.28 [1.11−1.48]	1.34 [1.12−1.60]	1.17 [0.91−1.50]	1.19 [0.97-1.46]	1.23 [0.95-1.58]	1.06 [0.75-1.52]	1.28 [1.11-1.48]	1.34 [1.12-1.60]	1.16 [0.91-1.49]
Vascular invasion	No	Yes	1.38 [1.18−1.62]	1.43 [1.19−1.73]	1.25 [0.93−1.66]	1.39 [1.13-1.72]	1.41 [1.09-1.83]	1.32 [0.92-1.91]	1.38 [1.18-1.62]	1.43 [1.18-1.73]	1.25 [0.94-1.66]
Mismatch repair	Proficient	Deficient	*	*	*	*	*	*	*	*	*

Supplementary Table 4: Baseline characteristics of patients with intended CAPOX duration of 6 versus 3 months, in total cohort and subcohort with available PROs.

	Intended 6 months		Intended 3 months	
	OS cohort n=2534	PROs cohort n=97	OS cohort n=1904	PROs cohort n=309
Pathological TNM stage				
High-risk stage II	153 (6.0%)	6 (6.1%)	119 (6.3%)	21 (6.8%)
Low-risk stage III	1128 (44.5%)	59 (60.2%)	688 (36.1%)	120 (38.8%)
High-risk stage III	1253 (49.4%)	33 (33.7%)	1097 (57.6%)	168 (54.4%)
Age Mean (SD)	62.8 (8.6)	61.9 (8.7)	63.2 (9.6)	62.2 (8.9)
Sex				
Male	1428 (56.4%)	61 (62.2%)	994 (52.2%)	174 (56.3%)
Female	1106 (43.6%)	37 (37.8%)	910 (47.8%)	135 (43.7%)
ASA physical status				
ASA1	628 (26.7%)	26 (27.1%)	336 (18.3%)	64 (21.1%)
ASA2	1473 (62.6%)	62 (64.6%)	1154 (63.0%)	199 (65.7%)
ASA3-4	252 (10.7%)	8 (8.3%)	342 (18.7%)	40 (13.2%)
Missing	181	2	72	6
Tumor sidedness				
Left	1429 (56.9%)	56 (58.3%)	1036 (55.0%)	183 (60.0%)
Right	1082 (43.1%)	40 (41.7%)	848 (45.0%)	122 (40.0%)
Missing	23	2	20	4
Prior malignancy				
No	2304 (90.9%)	88 (89.8%)	1686 (88.6%)	277 (89.6%)
Yes	230 (9.1%)	10 (10.2%)	218 (11.4%)	32 (10.4%)
Radical resection				
R0	54 (2.2%)	1 (1.0%)	27 (1.4%)	5 (1.6%)
R1 or R2	2447 (97.8%)	95 (99.0%)	1844 (98.6%)	300 (98.4%)
Missing	33	2	33	4
Differentiation grade				
Good-moderate	2013 (87.0%)	82 (86.3%)	1598 (89.2%)	254 (87.3%)
Poor-undifferentiated	300 (13.0%)	13 (13.7%)	194 (10.8%)	37 (12.7%)
Missing	221	3	112	18
Lymph nodes				
≥10 dissected	97 (3.8%)	98 (100%)	72 (3.8%)	297 (96.1%)
<10 dissected	2437 (96.2%)	0 (0%)	1832 (96.2%)	12 (3.9%)
Lymphatic invasion				
No invasion	806 (32.9%)	28 (30.1%)	670 (35.9%)	117 (38.1%)
Lymphatic invasion	1643 (67.1%)	65 (69.9%)	1196 (64.1%)	190 (61.9%)
Missing	85	5	38	2
Vascular invasion				
No invasion	1272 (70.7%)	53 (67.1%)	1356 (72.6%)	216 (70.4%)
Vascular Invasion	528 (29.3%)	26 (32.9%)	512 (27.4%)	91 (29.6%)
Missing	734 (29.0%)	19 (19.4%)	36 (1.9%)	2
Mismatch repair status				
Proficient	168 (12.7%)	5 (7.0%)	228 (14.5%)	37 (13.5%)
Deficient	1160 (87.3%)	66 (93.0%)	1349 (85.5%)	238 (86.5%)
Missing	1206	27	327	34

Supplementary Table 5: Chemotherapy-induced polyneuropathy (CIPN) 2 years after 6 versus 3 months of CAPOX, dichotomized (not at all or a bit vs quite or very much)

	6 months n=90	**3 months** n=276	**p-value**
CIPN total score			<0.001
Mean [95%CI]	26.2 [21.3-31.1]	16.5 [14.4-18.6]	
Sensory subscore			<0.001
Mean [95%CI]	27.5 [22.4-32.7]	17.3 [15.0-19.7]	
Tingling fingers or hands			0.26
not at all	36 (40.0%)	172 (62.3%)	
a bit	32 (35.6%)	54 (19.6%)	
quite	16 (17.8%)	37 (13.4%)	
very much	6 (6.7%)	13 (4.7%)	
Tingling toes or feet			0.0039
not at all	34 (37.8%)	149 (54.0%)	
a bit	22 (24.4%)	67 (24.3%)	
quite	22 (24.4%)	42 (15.2%)	
very much	12 (13.3%)	18 (6.5%)	
Numbness in fingers or hands			0.22
not at all	48 (53.3%)	193 (69.9%)	
a bit	29 (32.2%)	58 (21.0%)	
quite	12 (13.3%)	21 (7.6%)	
very much	1 (1.1%)	4 (1.4%)	
Numbness in toes or feet			0.035
not at all	40 (44.4%)	165 (59.8%)	
a bit	25 (27.8%)	65 (23.6%)	
quite	17 (18.9%)	36 (13.0%)	
very much	8 (8.9%)	10 (3.6%)	
Shooting or burning pain in fingers or hands			0.26
not at all	36 (40.0%)	172 (62.3%)	
a bit	32 (35.6%)	54 (19.6%)	
quite	16 (17.8%)	37 (13.4%)	
very much	6 (6.7%)	13 (4.7%)	
Shooting or burning pain in toes or feet			0.10
not at all	61 (67.8%)	221 (80.1%)	
a bit	15 (16.7%)	31 (11.2%)	
quite	7 (7.8%)	18 (6.5%)	
very much	7 (7.8%)	6 (2.2%)	
Difficulty in hearing			0.41
not at all	58 (64.4%)	209 (75.7%)	
a bit	24 (26.7%)	49 (17.8%)	
quite	6 (6.7%)	16 (5.8%)	
very much	2 (2.2%)	2 (0.7%)	
Motor subscore			0.0055
Mean [95%CI]	17.6 [13.4-21.8]	11.4 [9.6-13.2]	
Problem standing or walking due to difficulty feeling the ground			0.017
not at all	52 (57.8%)	220 (79.7%)	
a bit	26 (28.9%)	40 (14.5%)	
quite	8 (8.9%)	14 (5.1%)	
very much	4 (4.4%)	2 (0.7%)	
Difficulty in distinguishing between hot and cold water			0.30
not at all	72 (80.0%)	247 (89.5%)	
a bit	13 (14.4%)	22 (8.0%)	
quite	3 (3.3%)	6 (2.2%)	
very much	2 (2.2%)	1 (0.4%)	
Cramp in hands			0.78
not at all	65 (72.2%)	219 (79.3%)	

a bit	22 (24.4%)	44 (15.9%)	
quite	2 (2.2%)	8 (2.9%)	
very much	1 (1.1%)	5 (1.8%)	
Cramp in feet			0.23
not at all	64 (71.1%)	206 (74.6%)	
a bit	18 (20.0%)	57 (20.7%)	
quite	7 (7.8%)	9 (3.3%)	
very much	1 (1.1%)	4 (1.4%)	
Difficulity in holding a pen			0.75
not at all	67 (74.4%)	228 (82.6%)	
a bit	18 (20.0%)	37 (13.4%)	
quite	2 (2.2%)	9 (3.3%)	
very much	3 (3.3%)	2 (0.7%)	
Difficulty in manipulating small objects			<0.001
not at all	44 (48.9%)	201 (72.8%)	
a bit	25 (27.8%)	52 (18.8%)	
quite	17 (18.9%)	19 (6.9%)	
very much	4 (4.4%)	4 (1.4%)	
Difficulty in opening a jar or bottle			0.14
not at all	53 (58.9%)	160 (58.0%)	
a bit	20 (22.2%)	80 (29.0%)	
quite	14 (15.6%)	33 (12.0%)	
very much	3 (3.3%)	3 (1.1%)	
Feet dropping downwards			0.036
not at all	74 (82.2%)	247 (89.5%)	
a bit	8 (8.9%)	22 (8.0%)	
quite	5 (5.6%)	6 (2.2%)	
very much	3 (3.3%)	1 (0.4%)	
Difficulty climbing stairs or getting up out of a chair			0.025
not at all	54 (60.0%)	213 (77.2%)	
a bit	24 (26.7%)	46 (16.7%)	
quite	9 (10.0%)	15 (5.4%)	
very much	3 (3.3%)	2 (0.7%)	
Difficulty using pedals when driving a car			0.16
not at all	66 (88.0%)	222 (91.4%)	
a bit	8 (10.7%)	20 (8.2%)	
quite	1 (1.3%)	1 (0.4%)	
very much	0 (0%)	0 (0%)	
missing (not in motor core)	15 (16.7%)	33 (12.0%)	
Autonomic subscore			0.030
Mean [95%CI]	14.4 [9.6-19.3]	8.8 [7.0-10.6]	
Blurred vision			0.0076
not at all	67 (74.4%)	231 (83.7%)	
a bit	16 (17.8%)	41 (14.9%)	
quite	6 (6.7%)	3 (1.1%)	
very much	1 (1.1%)	1 (0.4%)	
Dizzy when standing up			0.27
not at all	62 (68.9%)	214 (77.5%)	
a bit	22 (24.4%)	53 (19.2%)	
quite	6 (6.7%)	9 (3.3%)	
very much	0 (0%)	0 (0%)	
Difficulty in having or maintaining an erection			0.16
not at all	15 (30.0%)	59 (42.1%)	
a bit	14 (28.0%)	39 (27.9%)	
quite	9 (18.0%)	21 (15.0%)	
very much	12 (24.0%)	21 (15.0%)	
missing (not in autonomic score)	40 (44.4%)	136 (49.3%)	

Supplementary Table 6: Quality of life (QoL) QLQ-C30 scores, functional scales, and symptoms two years after 6 versus 3 months of CAPOX.

	6 months n=94	**3 months** n=302	**p-value**
QoL Summary Score	80.9 [78.6-83.2]	83.9 [82.8-84.9]	0.013
Global Health Status	73.3 [69.2-77.5]	79.1 [77.2-81.1]	0.022
Physical Function	89.8 [86.8-92.8]	90.3 [88.6-92.0]	0.55
Role Function	78.4 [72.7-84.0]	85.4 [82.6-88.1]	0.017
Emotional Function	82.9 [79.2-86.6]	85.9 [83.9-87.9]	0.068
Cognitive Function	82.3 [77.8-86.8]	86.6 [84.6-88.6]	0.11
Social Function	81.6 [76.7-86.4]	89.7 [87.5-91.8]	<0.001
Fatigue	27.0 [21.9-32.0]	19.9 [17.6-22.3]	0.0093
Nausea and Vomiting	5.3 [2.5-8.2]	2.9 [1.6-4.1]	0.037
Pain	15.1 [10.5-19.7]	10.1 [8.0-12.2]	0.053
Dyspnea	13.1 [8.7-17.5]	11.4 [9.1-13.6]	0.58
Insomnia	21.3 [16.4-26.2]	18.1 [15.4-20.8]	0.18
Appetite Loss	9.2 [4.9-13.5]	4.5 [2.7-6.3]	0.016
Constipation	10.3 [6.5-14.0]	9.3 [7.0-11.5]	0.31
Diarrhea	16.3 [10.6-22.1]	11.0 [8.7-13.3]	0.25

Supplementary Table 7: Workability index (WAI) two years after 6 versus 3 months of CAPOX, pooled as indicated for $\chi 2$ test.

	6 months n=29	**3 months** n=91	**p-value**
WAI score (7-49)			0.12
Mean [95%CI]	31.5 [27.9-35.1]	35.3 [33.8-36.7]	
WAI category			0.25
poor (WAI score 7-27)	8 (27.6%)	10 (11.0%)	
moderate (WAI score 28-36)	8 (27.6%)	27 (29.7%)	
good (WAI score 37-43)	13 (44.8%)	53 (58.2%)	
excellent (WAI score 44-49)	0 (0%)	1 (1.1%)	
Current work ability compared to highest work ability ever (1-10)			0.042
Mean [95%CI]	6.6 [5.6-7.7]	7.6 [7.2-8.1]	
Current work ability with respect to the physical demands of work			0.063
very poor	2 (6.9%)	2 (2.2%)	
rather poor	2 (6.9%)	4 (4.4%)	
moderate		8 (8.8%)	
good	13 (44.8%)	48 (52.7%)	
very good	6 (20.7%)	29 (31.9%)	
Current work ability with respect to the mental demands of work			0.0098
very poor	2 (6.9%)	2 (2.2%)	
rather poor	3 (10.3%)	4 (4.4%)	
moderate	7 (24.1%)	13 (14.3%)	
good	11 (37.9%)	52 (57.1%)	
very good	6 (20.7%)	20 (22.0%)	

	6 months n=29	3 months n=91	p-value
Number of current diseases or injuries			
0	9 (60.0%)	40 (53.3%)	1
1	0 (0%)	2 (2.7%)	
2 or 3	1 (6.7%)	12 (16.0%)	
4 or more	5 (33.3%)	21 (28.0%)	
Estimated work impairment due to diseases			0.31
entirely unable to work	22 (75.9%)	53 (58.2%)	
able to do only part time work	1 (3.4%)	10 (11.0%)	
sometimes slow down work pace	6 (20.7%)	28 (30.8%)	
some symptoms	0 (0%)	0 (0%)	
no hindrance	0 (0%)	0 (0%)	
Number of whole days off work due to illness during last year			0.24
>100 days in year	5 (17.2%)	12 (13.2%)	
25-59 days in year	4 (13.8%)	10 (11.0%)	
10-24 days in year	2 (6.9%)	3 (3.3%)	
1-9 days in year	9 (31.0%)	22 (24.2%)	
0 days in year	9 (31.0%)	44 (48.4%)	
not applicable	0 (0%)	0 (0%)	
Expect to be able to do current job 2 years from now			0.094
unlikely	5 (17.2%)	5 (5.5%)	
maybe	6 (20.7%)	15 (16.5%)	
very likely	18 (62.1%)	71 (78.0%)	
Able to enjoy your regular daily activities in past 3 months			0.051
Never	0 (0%)	0 (0%)	
rather seldom	1 (3.4%)	0 (0%)	
sometimes	7 (24.1%)	5 (5.5%)	
rather often	10 (34.5%)	46 (50.5%)	
often	11 (37.9%)	40 (44.0%)	
Being active and alert in past 3 months			0.86
never	1 (3.4%)	0 (0%)	
rather seldom	4 (13.8%)	4 (4.4%)	
sometimes	4 (13.8%)	14 (15.4%)	
rather often	12 (41.4%)	45 (49.5%)	
often	8 (27.6%)	28 (30.8%)	
Feeling full of hope about the future in past 3 months			0.051
never	0 (0%)	0 (0%)	
rather seldom	1 (3.4%)	0 (0%)	
sometimes	7 (24.1%)	5 (5.5%)	
rather often	10 (34.5%)	46 (50.5%)	
often	11 (37.9%)	40 (44.0%)	

2a

Reply to putting IDEA's results into practice: practicality should rule complexity in stage III colon cancer

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We would like to compliment Samnani et al.[1] on their insightful evaluation of the impact of the IDEA studies on real-world practice. As requested in the accompanied editorial[2], we would hereby like to corroborate and enrich these findings with additional results from the Dutch population-based cancer registry. Using the Alberta Cancer Registry (ACR), the authors demonstrate a rapid and successful implementation of the IDEA trial results in clinical practice in Canada. When comparing patients with stage III colon cancer (CC) receiving adjuvant chemotherapy (ACT) pre-IDEA (2012-2018, n=460) versus post-IDEA (April 2018-2022, n=280), use of capecitabine + oxaliplatin (CAPOX) increased (23% to 51%) and median duration decreased (4.8 to 2.7 months). Moreover, this reduced duration was not at the expense of overall survival (OS) or cancer-specific survival. However, implementation varied across treatment centers, mirroring discrepancies in international guidelines.[3,4]

Especially in high-risk stage III (T4/N2) CC, oncologists are hesitant to reduce treatment duration due to concerns about undertreatment, as the IDEA trial did not prove non-inferiority of 3 months of infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX) in this group of patients.[5,6] In the editorial[2], Sanoff refers to the ACCENT group for additional data on this risk group, demonstrating that compared with 6 months of oxaliplatin-based doublet, stopping oxaliplatin at ≥50% of doses while continuing fluoropyrimidine was not associated with inferior OS or disease-free survival.[7] We can complement these data with the results of 6 months of CAPOX versus 3 months of CAPOX in daily clinical practice, as the Netherlands adopted 3 months of CAPOX also for high-risk stage III, directly after the presentation of the IDEA results.[8]

The Dutch guideline changed in 2017 from 6 months to 3 months of CAPOX, for patients with high-risk stage II (T4N0) and all stage III CC. Comparable with the ACR analysis, we used the Netherlands Cancer Registry (NCR) to analyze patients with CC treated with ACT pre-IDEA (2015-2016) versus post-IDEA (2018-2019). The preferred regimen in the Netherlands is CAPOX. Among patients receiving ACT, the proportion treated with CAPOX increased (74% to 83%), concomitant to shorter duration of CAPOX (18.6 to 9.5 weeks).[9] Multivariable analysis showed that 5-year OS was comparable between patients receiving 6 months of CAPOX pre-IDEA and 3 months of CAPOX post-IDEA, both across all high-risk stage II and stage III CC (n=2330, hazard ratio [HR] 1.02) and in the high-risk stage III subgroup (n=992, HR 1.06).[10]

Another subgroup of interest is based on older age and comorbidity, because of tolerance issues of especially oxaliplatin. In patients aged 75 years and older, we show that the guideline change to reduce duration of CAPOX (18.3 to 8.6 weeks) was associated with more elderly patients opting for CAPOX (27% to 49%).[9] Importantly across any age, shorter CAPOX duration was confirmed to reduce patient-reported long-term neurotoxicity and improve quality of life.[10] This is particularly relevant because half of patients is cured by the surgery alone and currently overtreated with ACT.[11]

In conclusion, the NCR data show quick implementation of the IDEA results in clinical practice, also for high-risk stage III, without compromising OS. Comparison with the ACR data reveals interesting and relevant international variations in adoption of trial results in guidelines and in clinical care. Part of these variations may be attributed to culturally defined differences in the preferences of patients and clinicians. Based on our real-world data results, we hereby support Sanoff's[2] assertion that "all people with stage III CC (regardless of substage) should be included in the decision as to whether their chemotherapy can stop after 3 months, thereby markedly decreasing their risk of long-term chemotherapy induced neuropathy and allowing for faster return to work and home functions."

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3

A comparison by mismatch repair status of real-world characteristics and outcomes in patients with stage II/III rectal cancer in the Netherlands

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Abstract

Introduction

A subset of rectal cancer (RC), <5%, exhibits mismatch repair deficiency (dMMR); the remaining are classified as proficient (pMMR). Reported evidence on differences between dMMR and pMMR RC is limited. In this nationwide Dutch study, we compared patients with dMMR and pMMR stage II/III RC based on patient and tumor characteristics, treatment patterns, and associated outcomes.

Methods

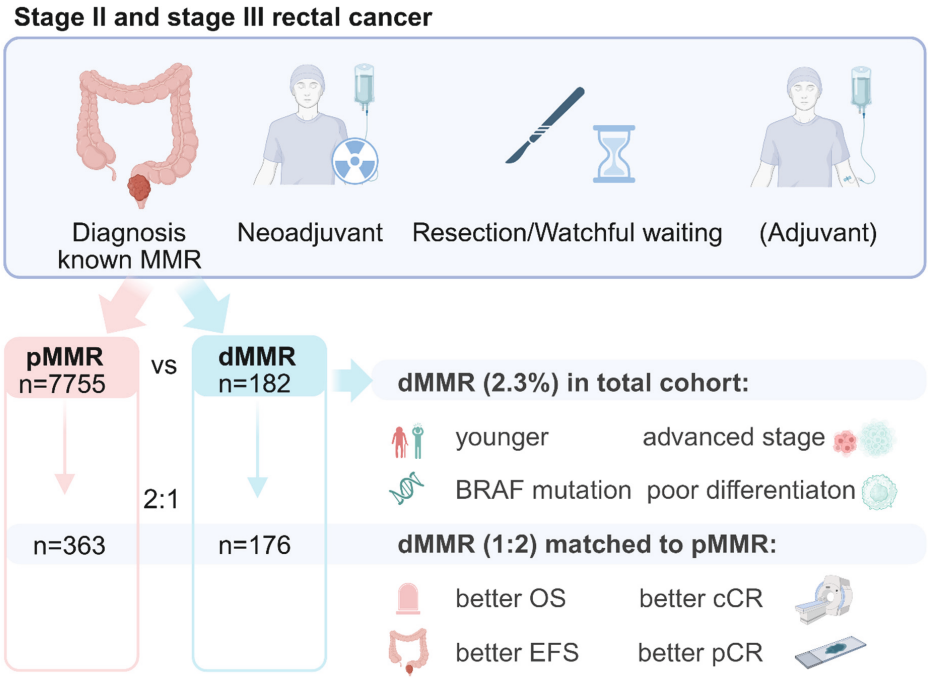
Patients diagnosed in 2015–2022 with known MMR status were selected from the Netherlands Cancer Registry. Demographic, tumor, and treatment characteristics were compared in the total cohort. Subsequently, dMMR patients were matched 1:2 to pMMR patients on age, year of diagnosis, clinical tumor stage and node stage. Overall survival (OS), and event-free survival (EFS) were analyzed using Kaplan–Meier estimates and Cox proportional hazard models.

Results

Among 7937 eligible patients, 182 (2.3%) had dMMR RC. These patients were younger and had more poorly differentiated tumors, more variation in histology, more advanced clinical stages, and more BRAF mutations. In the total cohort, OS was better for dMMR patients compared with pMMR patients (HR 0.62 [95%CI 0.43–0.91]). In the matched cohort, dMMR patients continued to exhibit improved OS (HR 0.54 [0.33–0.88]), and demonstrated improved EFS (HR 0.43 [0.29–0.63]) after adjustment for potential confounders.

Conclusion

dMMR RC is a rare entity associated with younger age and more advanced stages. Although patients with dMMR RC had significantly improved OS and EFS compared with patients with pMMR RC, immunotherapy may further enhance outcomes. This real-world study provides a basis for future investigations aimed at optimizing therapeutic strategies for patients with dMMR RC.


Stage II and stage III rectal cancer
Diagnosis known MMR
Neoadjuvant
Resection/Watchful waiting
(Adjuvant)
pMMR n=7755
vs
dMMR n=182
2:1
n=363
n=176
dMMR (2.3%) in total cohort:
younger
advanced stage
BRAF mutation
poor differentiaton
dMMR (1:2) matched to pMMR:
better OS
better cCR
better EFS
better pCR

Introduction

Tumors with deficient mismatch repair (dMMR) represent a distinct subgroup within colorectal cancer (CRC). dMMR tumors exhibit different biological behavior and treatment outcomes compared with proficient mismatch repair (pMMR) tumors.[1–3] dMMR is characterized by defects in the mismatch repair system resulting in high microsatellite instability (MSI), leading to accumulation of mutations and formation of neoantigens.[4,5] Neoantigens may be recognized by the immune system, rendering dMMR tumors susceptible to immunotherapy. In metastatic CRC, the treatment landscape for patients with a dMMR tumor (~5%) has fundamentally changed as immunotherapy studies have reported durable responses and improved survival.[6-9]

Also in non-metastatic colon cancer, dMMR tumors (~15–20%) demonstrate different clinical behavior compared with pMMR tumors.[10,11] Studies indicate that although patients with early stage dMMR colon cancer generally have a better prognosis compared with patients with a pMMR tumor, dMMR tumors seem to have a lack of benefit from adjuvant 5-fluorouracil monotherapy.[12–16] However, recent studies have shown that these dMMR tumors respond very well to neoadjuvant immunotherapy.[17]

In non-metastatic rectal cancer (RC), the incidence of dMMR tumors (<5%) is even lower, leading to limited knowledge about the biological behavior, response to treatment, and clinical outcomes within this subset of patients.[18,19] A trial by Cercek et al. has shown encouraging results following programmed cell death protein 1 (PD-1) blockade with dostarlimab for patients with stage II/III dMMR RC. After 6 months of neoadjuvant treatment, 100% of patients had a clinical complete response (cCR) allowing for organ preservation.[20] Twenty out of 41 patients achieved a sustained cCR with a median follow-up of 28.9 months. Although follow-up of recurrence-free survival (RFS) and overall survival (OS) is ongoing, these results suggest that patients with dMMR locally advanced RC may be highly responsive to immunotherapy.

Due to the rarity of dMMR in stage II/III RC, the number of patients in clinical studies is limited. This emphasizes the need for real-world data to contextualize findings from single-arm clinical studies. In this nationwide Dutch study, we aim to compare patients with dMMR and pMMR stage II/III RC by examining differences in patient and tumor characteristics, treatment patterns, and outcomes.

Methods

Study population

The study population was based on the nationwide Netherlands Cancer Registry (NCR), a comprehensive, population-based database that captures all newly diagnosed malignancies in the Netherlands with >95% coverage.[21] Tumor location is classified using the International Classification of Diseases for Oncology. Staging is recorded based on the Union for International Cancer Control Tumor Node and Metastasis (TNM) system, following the edition applicable at the time of diagnosis, which did not differ in T and N classification during the period of this study.

The vital status of patients is determined annually by linking the NCR to the Municipal Personal Records Database, which maintains records on the vital status of Dutch residents. Data on patient demographics, tumor characteristics, diagnosis, and treatment are routinely extracted from medical records by trained data managers of the Netherlands Comprehensive Cancer Organisation (IKNL). All adult patients with clinical stage II/III RC and known mismatch repair (MMR) status between 2015 and 2022 were included in this study. Patients who harbored a neuroendocrine or squamous cell tumor were excluded. Based on available data from the NCR, two different cohorts were defined as illustrated in Figure 1. The total cohort included all patients with a known MMR status in the NCR. A matched cohort was constructed, excluding patients without anticancer treatment, enabling the collection of additional highly detailed data on treatment patterns and outcomes. Patients with a dMMR tumor were matched in a 1:2 ratio to a subset of patients with a pMMR tumor, based on clinical T stage and N stage (exact matching), year of diagnosis (coarsened exact matching, allowing 1-year difference), and age (coarsened exact matching, categorized as <50, 50–70, or >70 years). These variables were chosen to minimize potential baseline confounding and allow for the comparison of more equivalent groups. Matching was conducted using the R package *Match-It*.

Statistical analyses and endpoints

Patient, tumor, and treatment characteristics were compared between all dMMR and pMMR patients in the total cohort. Continuous variables were analyzed using a two-sided unpaired t-test and categorical variables using the χ^2 test or Fisher's exact test. In the total cohort, OS was defined as the time from diagnosis to death from any cause, with subgroup analyses based on clinical stage. The matched cohort included only

patients who received anticancer treatment, facilitating analyses per treatment subgroup for OS and event-free survival (EFS). Patients were categorized into three different treatment groups according to standard Dutch clinical practice, including patients receiving 1) upfront surgery, 2) short-course radiotherapy, and 3) long-course neoadjuvant treatment. This treatment was indicated for, respectively, 1) low-risk–cT1-3abN0, 2) intermediate-risk–cT3cdN0 or cT1-3N1, 3) high-risk–cT4/cN2.

OS in the matched cohort was defined as the time from start of treatment to death from any cause. EFS was defined as the time from start of treatment to death from any cause, distant metastasis, or locoregional failure (LRF). LRF consisted of locally progressive disease leading to an unresectable tumor, an R2 resection, local recurrence after resection, or persistent disease after neoadjuvant treatment without salvage resection. LRF and distant metastasis were visualized by cumulative incidence curves and differences were tested by a Gray's test. OS and EFS curves were estimated using the Kaplan–Meier method and were compared using an univariable Cox proportional hazard regression model with a hazard ratio (HR) and 95% confidence interval (CI). A multivariable Cox proportional hazard analysis was conducted to assess OS and EFS in the matched cohort, adjusting for potential confounders that remained different between dMMR and pMMR patients after matching: age, sex, American Society of Anesthesiologists (ASA) score, differentiation grade, histological type and distance to anal verge. Missing differentiation grade, ASA score and distance to anal verge were imputed by multivariate imputation by chained equations using R packages *mice* and *ggmice*. The imputation model included the variables from the substantive analysis model, the survival outcome (the event indicator and the Nelson–Aalen estimate of cumulative hazard of death[22]), and the following auxiliary variables: performance status, clinical T stage, clinical N stage, and MMR status. Differences in OS at 12 months and EFS at 12 and 36 months between patients with a dMMR versus a pMMR tumor were tested by a Z-test.

In patients treated with long-course neoadjuvant therapy, differences between dMMR and pMMR tumors were evaluated in radiological response, pathological response, organ preservation rate, chance of organ preservation, and cCR at 12 months (definitions are noted in Supplementary Table 1). For all tests, p-values <0.05 were considered statistically significant. All analyses were performed using R studio (v4.4.0).

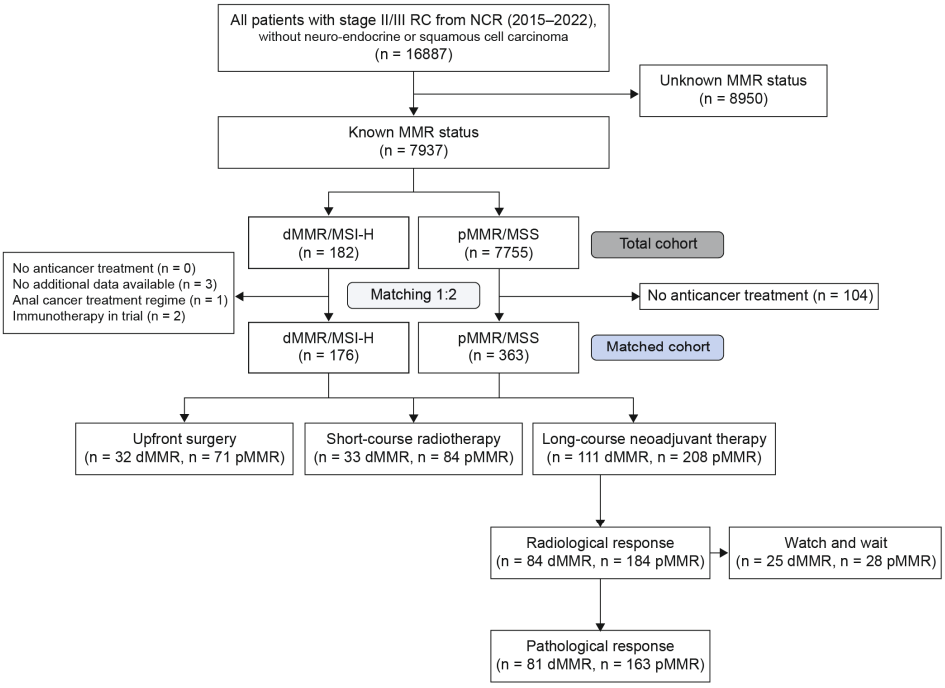


Figure 1: Flowchart of selected patients per cohort.
dMMR, deficient mismatch repair; MMR, mismatch repair; MSI, microsatellite instable; MSS, microsatellite stable; NCR, Netherlands Cancer Registry; pMMR proficient mismatch repair; RC, rectal cancer.

Table 1: Patient, tumor, and treatment characteristics according to mismatch repair status for the total cohort. *APR, abdominal peritoneal resection; ASA, American society of anesthesiologists; c, clinical; dMMR, deficient mismatch repair; IQR, interquartile range; LAR, low anterior resection; MRF, mesorectal fascia; N, node; pMMR, proficient mismatch repair; T, tumor; WHO, World Health Organization.*

	dMMR (n=182)	pMMR (n=7755)	p-value
Age Median (IQR)	57.0 (19.0%)	61.0 (13.0%)	<0.001
Sex			0.115
Male	103 (56.6%)	4855 (62.6%)	
Female	79 (43.4%)	2900 (37.4%)	
Year of diagnosis			0.464
2015	12 (6.6%)	395 (5.1%)	
2016	28 (15.4%)	995 (12.8%)	
2017	31 (17.0%)	1208 (15.6%)	
2018	21 (11.5%)	1244 (16.0%)	
2019	21 (11.5%)	1125 (14.5%)	
2020	22 (12.1%)	895 (11.5%)	
2021	30 (16.5%)	1060 (13.7%)	
2022	17 (9.3%)	833 (10.7%)	
WHO performance status			0.707
0	85 (66.9%)	3836 (68.3%)	
1	39 (30.7%)	1498 (26.7%)	
2	3 (2.4%)	228 (4.1%)	
3	0 (0%)	47 (0.8%)	
4	0 (0%)	7 (0.1%)	
Missing	55	2139	
ASA score			0.391
Class I	33 (20.4%)	1341 (18.7%)	
Class II	103 (63.6%)	4612 (64.4%)	
Class III–IV	26 (16.0%)	1206 (16.8%)	
Missing	20	596	
cT stage			0.009
T1	1 (0.5%)	57 (0.7%)	
T2	18 (9.9%)	638 (8.2%)	
T3	115 (63.2%)	5766 (74.4%)	
T4	43 (23.6%)	1135 (14.6%)	
Tx	5 (2.7%)	159 (2.1%)	
cN stage			0.002
N0	44 (24.2%)	2363 (30.5%)	
N1	59 (32.4%)	3043 (39.2%)	
N2	77 (42.3%)	2295 (29.6%)	
Nx	2 (1.1%)	54 (0.7%)	
BRAF mutation			<0.001
Wild type	24 (80.0%)	401 (93.3%)	
Mutant	6 (20.0%)	29 (6.7%)	
Missing	152	7325	
RAS mutation			0.061
Wild type	11 (64.7%)	231 (50.5%)	
Mutant	6 (35.3%)	226 (49.5%)	

Missing	165	7298	
Differentiation grade			<0.001
Good	4 (2.4%)	103 (1.4%)	
Moderate	127 (77.4%)	6668 (92.5%)	
Poor	33 (20.1%)	438 (6.1%)	
Anaplastic	0 (0%)	3 (0.0%)	
Missing	18	543	
Histology			<0.001
Adeno	157 (86.3%)	7340 (94.6%)	
Mucinous	18 (9.9%)	360 (4.6%)	
Signet ring cell	4 (2.2%)	48 (0.6%)	
Medullary type	2 (1.1%)	0 (0%)	
Adenosquamous	1 (0.5%)	2 (0.0%)	
Unknown	0 (0%)	5 (0.1%)	
MRF involvement			0.297
Yes	57 (44.9%)	2376 (41.3%)	
No	70 (55.1%)	3378 (58.7%)	
Missing	55	2001	
Distance to anal verge			0.090
0–5 cm	94 (54.7%)	4049 (53.8%)	
5–10 cm	55 (32.0%)	2465 (32.7%)	
10–15 cm	23 (13.4%)	878 (11.7%)	
>15 cm	0 (0%)	139 (1.8%)	
Missing	10	224	
Extramural invasion			<0.001
≤5 mm	51 (35.9%)	2273 (48.1%)	
>5 mm	39 (27.5%)	1169 (24.7%)	
Inapplicable	52 (36.6%)	1282 (27.1%)	
Missing	40	3031	
Treatment type			0.021
Neoadjuvant treatment only	36 (19.8%)	1076 (13.9%)	
Neoadjuvant treatment and resection	114 (62.6%)	4732 (61.0%)	
No neoadjuvant (upfront resection)	32 (17.6%)	1843 (23.8%)	
No treatment received	0 (0%)	104 (1.3%)	
Resection of primary tumor			0.113
No	36 (19.8%)	1180 (15.2%)	
Yes	146 (80.2%)	6575 (84.8%)	
Type of resection			0.669
APR	37 (20.3%)	1794 (23.1%)	
Other than APR (e.g. LAR)	109 (59.9%)	4781 (61.7%)	
Neoadjuvant treatment type			<0.001
Chemoradiation (± systemic therapy)	104 (57.1%)	3498 (45.1%)	
Radiotherapy only	34 (18.7%)	2032 (26.2%)	
Radiotherapy + systemic therapy	8 (4.4%)	237 (3.1%)	
Chemotherapy only	2 (1.1%)	25 (0.3%)	
Targeted therapy	2 (1.1%)	16 (0.2%)	
No neoadjuvant treatment	32 (17.6%)	1947 (25.1%)	
Adjuvant treatment			1
No	172 (94.5%)	7317 (94.4%)	
Yes	10 (5.5%)	438 (5.6%)	

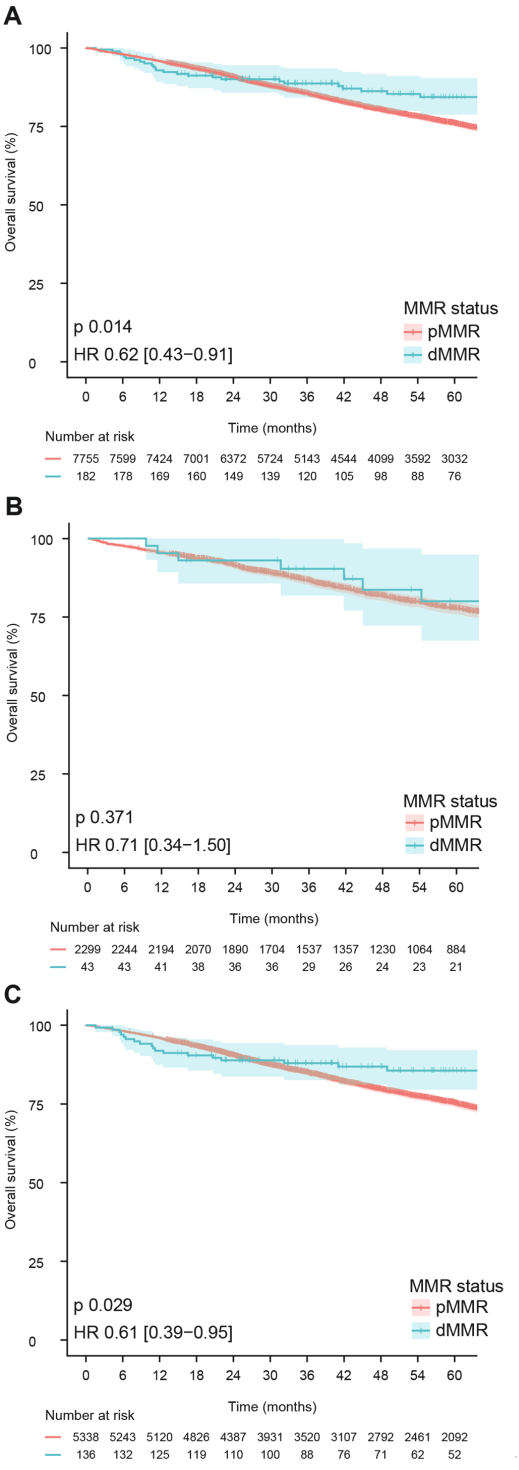
Results

Differences in patient, tumor and treatment characteristics and OS in total cohort

Among 7937 patients with stage II/III RC diagnosed between 2015 and 2022 in the Netherlands with a known MMR status, 182 (2.3%) were dMMR and 7755 (97.7%) were pMMR (Figure 1). Patients with a dMMR tumor were significantly younger than patients with a pMMR tumor (median age 57.0 versus 61.0 years; Table 1). No significant differences were observed in sex, year of diagnosis, performance status, or ASA score. Compared with patients with pMMR RC, patients with dMMR RC were characterized by a higher rate of poorly differentiated tumors (20% versus 6%), more mucinous or signet cell adenocarcinoma histological type (12% versus 5%), and a higher frequency of *BRAF* mutations (20% versus 7%). Additionally, dMMR tumors were more frequently diagnosed at an advanced clinical stage (cT4B 17% versus 9%, cN2 42% versus 30%), consistent with subtle differences in treatment patterns between dMMR and pMMR tumors.

Neoadjuvant treatment followed by resection was the predominant treatment approach regardless of MMR status (63% in dMMR versus 61% in pMMR). However, patients with a dMMR tumor were slightly more likely to receive neoadjuvant treatment only (20% versus 14%) and less likely to undergo upfront resection (18% versus 24%). Additionally, the type of neoadjuvant treatment differed: patients with a dMMR tumor were more likely to receive chemoradiation (57.1% versus 45.1%) and less likely to receive only radiotherapy (18.7% versus 26.2%). (Neo)adjuvant immunotherapy was not registered in clinical practice in this period in the Netherlands. OS differed between dMMR and pMMR, with better outcomes for dMMR (HR 0.62 [95%CI 0.43–0.91]; Figure 2A). A subgroup analysis per clinical stage revealed a significantly better OS for dMMR stage III (n=136) compared with pMMR stage III patients (n=5338) (HR 0.61 [0.39–0.95]; Figure 2B). However, this difference was not observed in dMMR stage II (n=43) versus pMMR stage II patients (n=2299) (HR 0.71 [0.34–1.5]; Figure 2C).

Figure 2: Kaplan–Meier OS analyses according to mismatch repair status. A) OS for the total cohort; B) OS for stage II patients in the total cohort; C) OS for stage III patients in the total cohort.
CI, confidence interval; dMMR, deficient mismatch repair; HR, hazard ratio; MMR, mismatch repair; OS, overall survival;


A
Overall survival (%)
100
75
50
25
0
p 0.014
HR 0.62 [0.43−0.91]
MMR status
pMMR
dMMR
0 6 12 18 24 30 36 42 48 54 60
Time (months)
Number at risk
7755 7599 7424 7001 6372 5724 5143 4544 4099 3592 3032
182 178 169 160 149 139 120 105 98 88 76
B
Overall survival (%)
100
75
50
25
0
p 0.371
HR 0.71 [0.34−1.50]
MMR status
pMMR
dMMR
0 6 12 18 24 30 36 42 48 54 60
Time (months)
Number at risk
2299 2244 2194 2070 1890 1704 1537 1357 1230 1064 884
43 43 41 38 36 36 29 26 24 23 21
C
Overall survival (%)
100
75
50
25
0
p 0.029
HR 0.61 [0.39−0.95]
MMR status
pMMR
dMMR
0 6 12 18 24 30 36 42 48 54 60
Time (months)
Number at risk
5338 5243 5120 4826 4387 3931 3520 3107 2792 2461 2092
136 132 125 119 110 100 88 76 71 62 52

Differences in OS and EFS in the matched cohort

The matched cohort consists of 176 patients with a dMMR tumor matched to 363 patients with a pMMR tumor, all receiving anticancer treatment (Figure 1). Differentiation grade, histological type, and *BRAF* status remained significantly different between dMMR and pMMR tumors after matching (Supplementary Table 2). No significant differences in treatment patterns were seen after matching between dMMR and pMMR groups. Neoadjuvant treatment (chemoradiation +/- systemic therapy) followed by resection was most common in both groups. The percentage of patients receiving a stoma was comparable for dMMR and pMMR groups (64.2% in both groups; Supplementary Table 2), as was the permanent stoma rate at 12 months (35.2% and 34.7%, respectively). OS was compared between dMMR and pMMR tumors in the overall matched cohort (Figure 3A) and in subgroups of patients with stage II disease as well as stage III disease (Supplementary Figure 1A and 1B). MMR status was independently associated with OS in the multivariable analysis (HR 0.54 [CI 0.33–0.88]; Table 2).

In multivariable analyses, a significantly better EFS was found for patients with a dMMR tumor compared with patients with a pMMR tumor (HR 0.43 [0.29–0.63]; Figure 3B; Table 2). While the 12-month EFS rates were comparable between the two groups (85.7% and 81.5%, p=0.21), the 36-month EFS was significantly higher for patients with a dMMR tumor (79.6% and 64.7%, p<0.001). The difference in EFS was more pronounced for stage III versus stage II disease (Supplementary Figure 1C and 1D).

When evaluating patterns of recurrence, the cumulative incidences of locoregional failure were comparable between dMMR (10% [6.0–16%]) and pMMR tumors (12% [8.8–16], p=0.6) 3 years after start of treatment (Figure 3D). At 3 years, the cumulative probability of distant metastases was 11% (6.6–17%) for dMMR and 29% (24–34%) for pMMR tumors (p<0.001; Figure 3C). Notably, dMMR tumors were more likely to show first recurrence in the peritoneum (23.5% versus 7.1%) and less likely to have multiple metastatic sites (17.6% versus 29.3%; Supplementary Table 3).

Survival after recurrence differed between dMMR and pMMR tumors, with worse 1-year OS in 26 patients with a recurrent dMMR tumor (73.1% [57.9–92.3]) compared with 122 pMMR patients (95.9% [92.5–99.5], p=0.01; Supplementary Figure 2).

Table 2: OS and EFS by mismatch repair status (matched cohort) in univariable and multivariable models adjusted for sex, age, ASA score, differentiation grade, histological type and distance to anal verge in stage II/III rectal cancer. pMMR was selected as the reference, unknown ASA score and differentiation grade and distance to anal verge were imputed by MICE with Nelson–Aalen estimator.
CI, confidence interval; dMMR, deficient mismatch repair; EFS, event-free survival; HR, hazard ratio; MICE, multivariate imputation by chained equations; OS, overall survival; pMMR, proficient mismatch repair.

	Univariable analysis			Multivariable analysis		
	HR	95% CI	p-value	HR	95% CI	p-value
OS dMMR	0.73	0.47–1.16	0.183	0.54	0.33–0.88	0.016
EFS dMMR	0.54	0.38–0.77	<0.001	0.43	0.29–0.63	<0.001

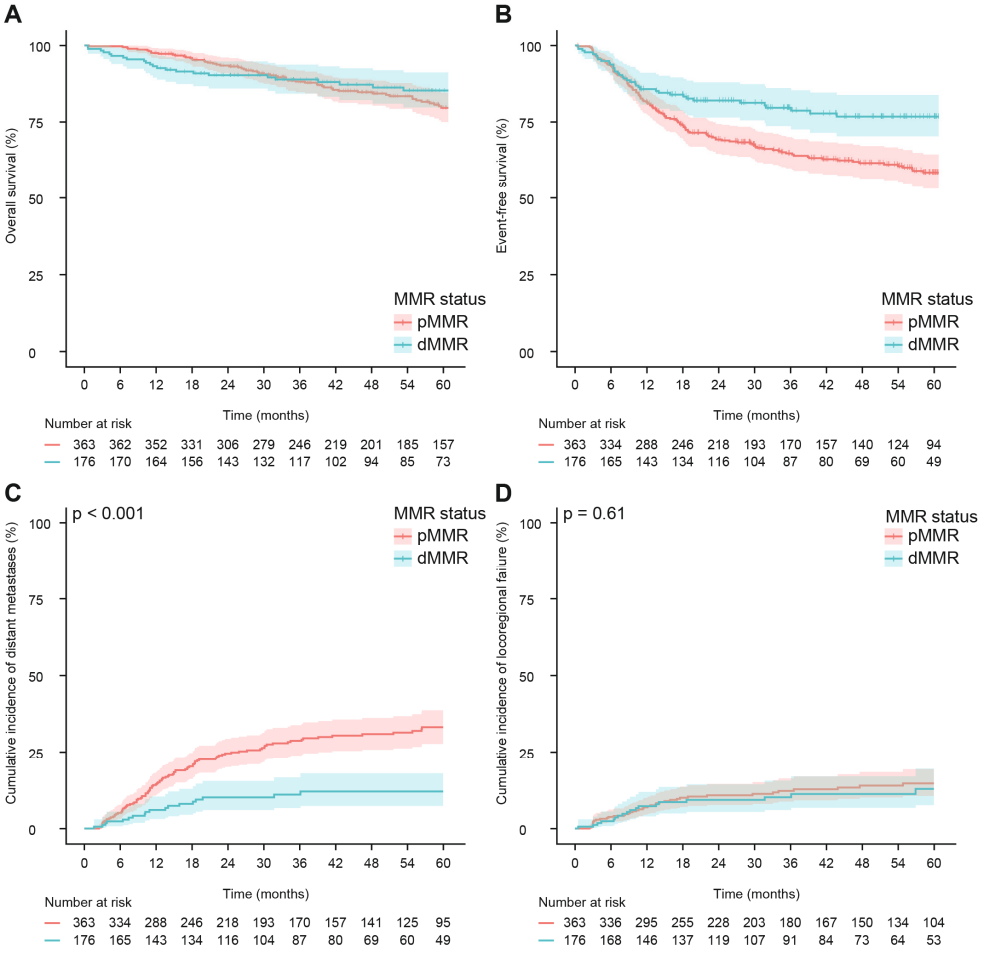


Figure 3: Kaplan–Meier analyses according to MMR status in the matched cohort. A) OS; B); EFS; C) Cumulative incidence of distant metastases; D) Cumulative incidence of locoregional failure. *dMMR, deficient mismatch repair; EFS, event-free survival; MMR, mismatch repair; OS, overall survival; pMMR, proficient mismatch repair.*

Differences in outcome after upfront surgery or short-course radiotherapy

Upfront surgery was given in 32 (18%) patients with a dMMR tumor and in 71 (20%) patients with a pMMR tumor in the matched cohort. There was no significant difference in OS between dMMR and pMMR tumors in this subgroup (HR 0.47 [0.16–1.42]; Supplementary Figure 3A). Patients with a dMMR tumor with upfront surgery exhibited superior EFS compared with patients with a pMMR tumor with upfront surgery (HR 0.31 [0.11–0.9]; Supplementary Figure 3B). Furthermore, 33 (19%) patients with a dMMR tumor and 84 (23%) patients with a pMMR tumor received short-course radiotherapy, which was followed by a resection in 28/33 (85%) patients with a dMMR and in 77/84 (92%) patients with a pMMR tumor. In this subgroup of patients, OS (HR 1.22 [0.5–2.93]; Supplementary Figure 3C) and EFS (HR 0.65 [0.29–1.49]; Supplementary Figure 3D) were not significantly different between dMMR and pMMR tumors.

Differences outcome after long-course neoadjuvant treatment in the matched cohort

In the matched cohort, most patients (111 (63%) dMMR and 208 (57%) pMMR) received long-course neoadjuvant treatment, allowing for the evaluation of neoadjuvant treatment response and the proportion of patients managed with a watch-and-wait strategy. OS did not differ significantly between dMMR and pMMR tumors in this treatment subgroup (HR 0.69 [0.38–1.28]; Supplementary Figure 3E). However, patients with a dMMR tumor treated with neoadjuvant therapy showed a significantly lower risk of events compared with patients with a pMMR tumor (HR 0.56 [0.36–0.86]; Supplementary Figure 3F).

Radiological response to neoadjuvant therapy was also evaluable for 84 patients with a dMMR tumor and 184 patients with a pMMR tumor within this subgroup. Clinical complete response was seen in 21.4% of patients with a dMMR tumor and in 9.2% of patients with a pMMR tumor and partial response in 69.0% versus 78.3%, respectively (p=0.067) (Table 3B). Neoadjuvant treatment was followed by resection in 82 (74%) patients with a dMMR tumor and 167 (80%) patients with a pMMR tumor (Table 3A). Pathological responses could be evaluated in 81 patients with a dMMR tumor and in 163 patients with a pMMR tumor, showing pathological complete response (pCR/ypT0N0) in 25 (30.9%) of the patients with dMMR tumors and in 20 (12.3%) of the patients with pMMR tumors (p<0.001) (Table 3C).

Table 3. Neoadjuvant treatment responses of patients in the matched cohort (excluding SCRT only) according to MMR status.
APR, abdominoperineal resection; deficient dMMR, mismatch repair; LAR, low anterior resection; pCR, pathological complete response; pMMR, proficient mismatch repair; SCRT, short-course radiotherapy.

	dMMR	pMMR	p-value
A) Long-course neoadjuvant treatment	**n=111**	**n=208**	
Treatment type			0.239
Neoadjuvant treatment only	29 (26.1%)	41 (19.7%)	
Neoadjuvant treatment and resection	82 (73.9%)	167 (80.3%)	
Neoadjuvant treatment			0.708
Chemoradiation (± systemic therapy)	100 (90.1%)	190 (91.3%)	
Radiotherapy only (long course)	1 (0.9%)	3 (1.4%)	
Radiotherapy ± systemic therapy	8 (7.2%)	14 (6.7%)	
Chemotherapy only	1 (0.9%)	1 (0.5%)	
Targeted therapy	1 (0.9%)	0 (0%)	
Resection type			0.495
Other than APR (e.g. LAR)	54 (48.6%)	101 (48.6%)	
APR	28 (25.2%)	66 (31.7%)	
Missing	29 (26.1%)	41 (19.7%)	
Adjuvant treatment			0.470
No	103 (92.8%)	197 (94.7%)	
Yes	8 (7.2%)	11 (5.3%)	
B) Available radiological response	**n=84**	**n=184**	
Radiological response			0.067
Complete response	18 (21.4%)	17 (9.2%)	
Partial response	58 (69.0%)	144 (78.3%)	
Mixed response	1 (1.2%)	2 (1.1%)	
Stable disease	2 (2.4%)	9 (4.9%)	
Progressive disease	2 (2.4%)	9 (4.9%)	
Unknown	3 (3.6%)	3 (1.6%)	
C) Available pathological response	**n=81**	**n=163**	
Tumor regression grade (Mandard)			0.007
Full regression	25 (30.9)	22 (13.5%)	
Moderate regression	46 (56.8)	115 (70.6%)	
Minor regression	2 (2.5)	3 (1.8%)	
No signs of regression	6 (7.4)	9 (5.5%)	
Missing	2 (2.5)	14 (8.6%)	
Pathological complete response			<0.001
pCR	25 (30.9)	20 (12.3%)	
No pCR	56 (69.1)	143 (87.7%)	

Most patients treated with long-course neoadjuvant treatment received chemoradiation, which was given in 100/111 (90%) patients with a dMMR tumor and in 190/208 (91%) patients with a pMMR tumor (Table 3A). A subgroup analysis of these patients showed clinical complete response in 22.4% of dMMR tumors and in 8.2% of pMMR tumors, and partial response was seen in 69.7% versus 79.4%, respectively (p=0.023; Supplementary Figure 4A). Of the 72 patients with a dMMR tumor and 152 patients with a pMMR tumor with evaluable pathological response, 23 (31.9%) and 18 (11.8%), respectively, showed a pCR (p <0.001; Supplementary Figure 4B). The EFS was significantly better for patients with dMMR tumors compared with pMMR tumors in this chemoradiation subgroup (HR 0.45 [0.27–0.73]; p<0.001; Supplementary Figure S4C). OS showed a numerically similar HR advantage for patients with a dMMR tumor, though this did not meet statistical significance (HR 0.49 [0.24–1.02]; p=0.051; Supplementary Figure 4D).

Long-course neoadjuvant treatment was not followed by resection in 29/111 (26%) patients with a dMMR tumor and 41/208 (20%) patients with a pMMR tumor (Table 3A). Among these patients, 4 (14%) with a dMMR tumor and 13 (32%) with a pMMR tumor did not undergo resection due to progression of disease during neoadjuvant treatment. A watch-and-wait strategy was explicitly chosen in the remaining 25 (86%) patients with a dMMR tumor and in 28 (68%) patients with a pMMR tumor (p=0.056), of whom 21 (84%) and 16 (62%) maintained a cCR at least 12 months after the end of neoadjuvant treatment (p=0.120). In the other patients, local recurrence (3 dMMR and 9 pMMR) or distant metastases (1 dMMR and 1 pMMR) were observed. When combining the watch-and-wait patients (25 dMMR and 28 pMMR) with patients showing pCR after resection (25 dMMR and 20 pMMR), organ preservation may theoretically have been achievable in 50 out of 111 neoadjuvant-treated patients with a dMMR tumor (45%), which is significantly higher than the 48 out of 208 pMMR with a pMMR tumor (23%, p<0.001).

Discussion

This retrospective, real-world cohort study describes differences in patient, tumor, and treatment characteristics and their associated outcomes in patients with dMMR compared with pMMR stage II/III RC. We observed significant differences in patient and tumor characteristics by MMR status in the total cohort of all patients diagnosed with RC. In a matched cohort, we demonstrated that patients with a dMMR tumor had a lower risk of death as well as a reduced risk of an event compared with patients with a pMMR tumor.

Firstly, the total cohort was analyzed to compare differences in patient, tumor, and treatment characteristics. Patients with a dMMR tumor in the total cohort were significantly younger and had more clinically advanced stages, more poorly differentiated tumors, more mucinous and signet ring cell adenocarcinomas, and more *BRAF* mutations compared with those with a pMMR tumor, mirroring findings from previous studies.[4,23–25] These distinct characteristics underscore the unique biological behavior of dMMR tumors in RC.

Secondly, an analysis of OS in the total cohort showed significantly better outcomes for patients with a dMMR tumor in comparison to patients with a pMMR tumor. Prior research investigating the prognostic effect of MMR status in patients with RC shows similar results. For example, Colombino et al.[26] demonstrated that patients with dMMR RC had better OS than those with pMMR RC, although their study included only patients undergoing upfront surgery. Similarly, Chen et al.[24] reported better OS rates for dMMR tumors among matched patients. However, these findings should be interpreted cautiously due to the small number (range 17–29) of dMMR tumors.

In the matched cohort, consistent with the total cohort, an improved OS was demonstrated within dMMR tumors in a multivariable analysis. Additionally, an EFS analysis in the matched cohort showed that patients with a dMMR tumor exhibit a significantly lower risk of an event compared with patients with a pMMR tumor. This finding aligns with existing literature reporting better RFS and disease-free survival (DFS) in patients with dMMR RC.[23,24] For instance, Meillan et al.[23] reported longer RFS in a cohort of 23 patients with a dMMR tumor treated with chemoradiation.

In the subgroup of patients with stage III disease, our study demonstrated that dMMR is associated with prolonged EFS. This prognostic benefit was not observed in the subgroup of patients with stage II disease. These findings should be interpreted with caution, due to the low number of patients with stage II dMMR tumors (n=41) limiting statistical power; as such, the results should be considered exploratory and hypothesis-generating rather than definitive. In colon cancer, dMMR has been associated with improved DFS in stage II but poorer DFS in stage III disease.[27,28] Notably, patients with stage II dMMR colon cancer appear to derive limited benefit from adjuvant 5-fluorouracil chemotherapy.[12–16] For RC, evidence remains scarce, but a recent study similarly reported no benefit of adjuvant treatment in dMMR RC patients following surgery.[29] Our findings suggest that dMMR rectal tumors on the other hand respond better to neoadjuvant chemoradiation compared to patients with a pMMR tumor. This may reflect an increased radiosensitivity of dMMR tumors[23,30], as well as the immunogenic effect of neoadjuvant treatment timing.[31] Specifically, neoadjuvant chemoradiation may stimulate immune activation in dMMR tumors, enhancing treatment response, whereas adjuvant therapies might delay this effect and potentially increase toxicity.

Building on these observations, our study also highlights the differential treatment responses associated with MMR status. Our findings indicate that patients with a dMMR tumor respond better to neoadjuvant therapy, in terms of cCR and pCR rates compared with patients with a pMMR tumor. This superior response was particularly pronounced in the subgroup of patients treated with chemoradiation, aligning with prior studies that have also demonstrated poor responses to neoadjuvant chemotherapy alone in patients with a dMMR tumor.[23–25,32] However, literature regarding the association between MMR status and response to neoadjuvant therapy in patients with RC remains inconsistent. For example, no significant differences in pCR rates based on MMR status following chemoradiation were found in two previous studies.[32,33] Acar et al.[34] and Hasan et al.[35] reported a decreased likelihood of achieving pCR in patients with a dMMR tumor after chemoradiation, findings that contradict our observations. In our study, the chance of organ preservation (cCR and pCR) was 45% in patients with dMMR RC and 23% in pMMR RC.

To our knowledge, this study represents the largest to date cohort to examine patient, tumor, and treatment characteristics in patients with dMMR RC, as well as their responses to treatment. The substantial inclusion of dMMR tumors is a key strength, providing a robust dataset for analysis despite the rarity of dMMR. The prevalence of dMMR in our total cohort was 2.3%, aligning with existing literature.[18,19]

Nonetheless, this study has inherent limitations, the most notably being selection bias. This bias arises from the inclusion of only patients with known MMR status, representing approximately half of all patients with RC in the NCR with stage II/III diagnosed between 2015 and 2022 (n=16887). MMR status was predominantly unknown in earlier incidence years of this study, as routine testing was gradually implemented in the Netherlands starting in 2015. Moreover, patients with known MMR status are typically younger, as the Dutch guidelines recommend MMR testing primarily for individuals under 70 years of age. Consequently, this study cohort may represent a fitter population that received different treatments compared with the total Dutch stage II/III RC population, potentially influencing prognostic outcomes. An additional limitation is the potential for clinical overstaging of dMMR tumors, given their association with increased immune infiltration. This may affect the accuracy of matching based on clinical T and N stages and could introduce a bias in favor of better outcomes for dMMR tumors, especially in the stage III subgroup analyses.[36] Given the smaller sample size, analyses of differences in response to neoadjuvant treatment within the matched cohort were not adjusted using a multivariable model. In the multivariable analyses of OS and EFS, we adjusted for the confounding effects of age, sex, histological type, and differentiation grade. Given the direction of these confounding effects, we expect that adjusting for these covariates would have enhanced our estimate. Lastly, the high proportion of patients with unknown *RAS* or *BRAF* mutational status, as well as the absence of data on germline testing to distinguish between hereditary (Lynch syndrome) and sporadic dMMR, prevented the inclusion of these molecular characteristics as covariates in the multivariable analyses. This may have limited the comprehensiveness of the adjusted models and the ability to explore etiologic subtypes.

Our study highlights favorable outcomes for stage II/III dMMR RCs. Nonetheless, a significantly higher risk of mortality was observed among patients with a dMMR tumor who experienced recurrence of disease within the first year of treatment initiation compared with patients with a pMMR tumor. Future research should therefore focus on identifying specific subgroups of dMMR patients at higher risk of recurrence, enabling development of targeted preventive strategies.

In our real-world observational data from a pre-immunotherapy era, where treatment outcomes primarily depended on response to chemoradiation, cCR without resection was limited to 23% in dMMR and 14% in pMMR, although pCR at resection was observed in another 31% in dMMR and 12% in pMMR. Whereas a complete response may allow for organ preservation, this was not the primary intention of treatment for most patients with RC treated in clinical practice between 2015 and 2022. As such, the cCR rate cannot be reliably assessed in this study. In cohorts with a specific focus on achieving organ preservation, cCR rates may have been higher, owing to a longer waiting period following chemoradiation, which would allow for tumor downstaging and potentially a cCR. With a primary focus on organ preservation, Cercek et al.,[20] reported 100% cCR in 41 patients with dMMR RC completing neoadjuvant treatment with the PD-1 checkpoint inhibitor dostarlimab. These findings underscore the potential for ICI to substantially improve clinical outcomes in patients with stage II/III dMMR RC.

In conclusion, this study represents the largest to date cohort to examine characteristics and outcomes by MMR status in patients with RC. dMMR RC is a rare entity characterized by younger patients and more advanced clinical stages. After matching for these variables and adjusting for additional confounders, patients with a dMMR tumor demonstrated significantly improved OS and EFS compared with patients with a pMMR tumor. Additionally, dMMR is associated with higher rates of cCR and pCR in this study, although superior outcomes may be achievable with immunotherapy. To facilitate accurate comparisons of emerging immunotherapies with the standard of care, accurately matched synthetic control arms are essential. This real-world data study provides a basis for future investigations aimed at optimizing therapeutic strategies for patients with dMMR RC.

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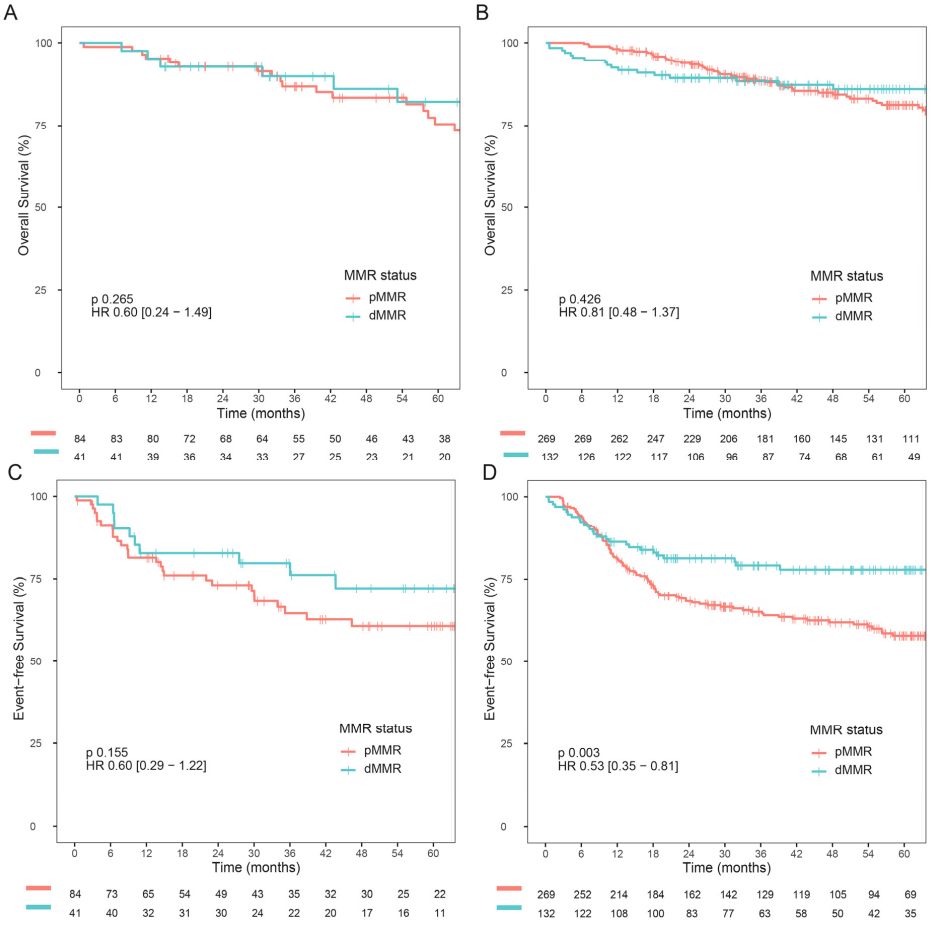
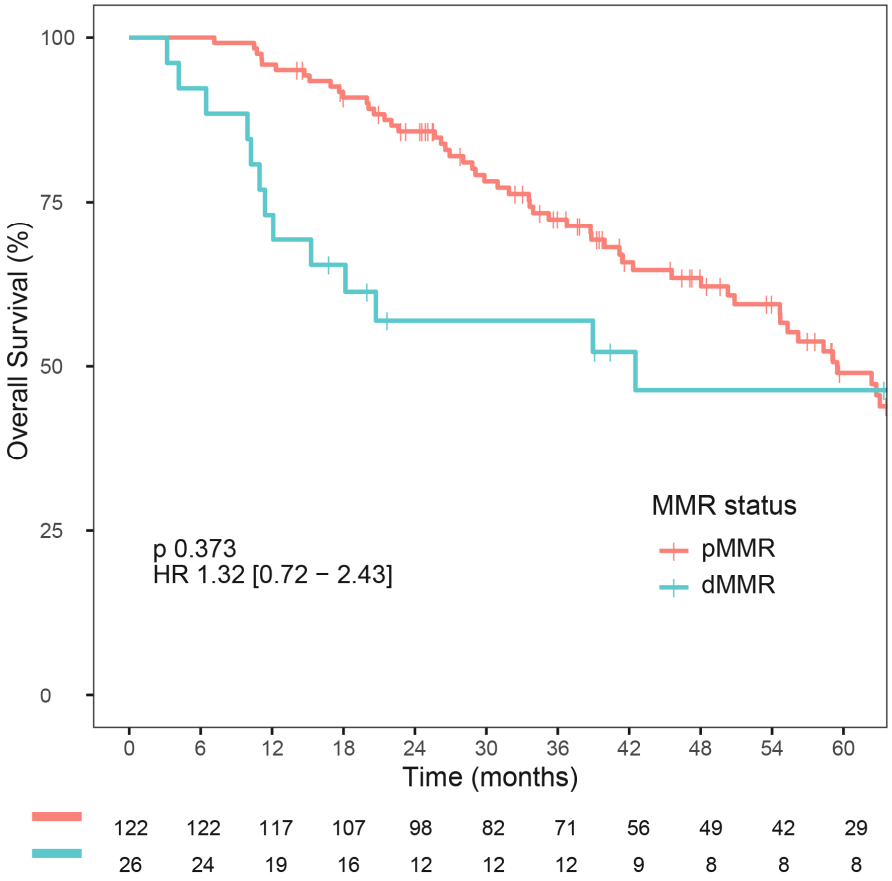
Supplementary material

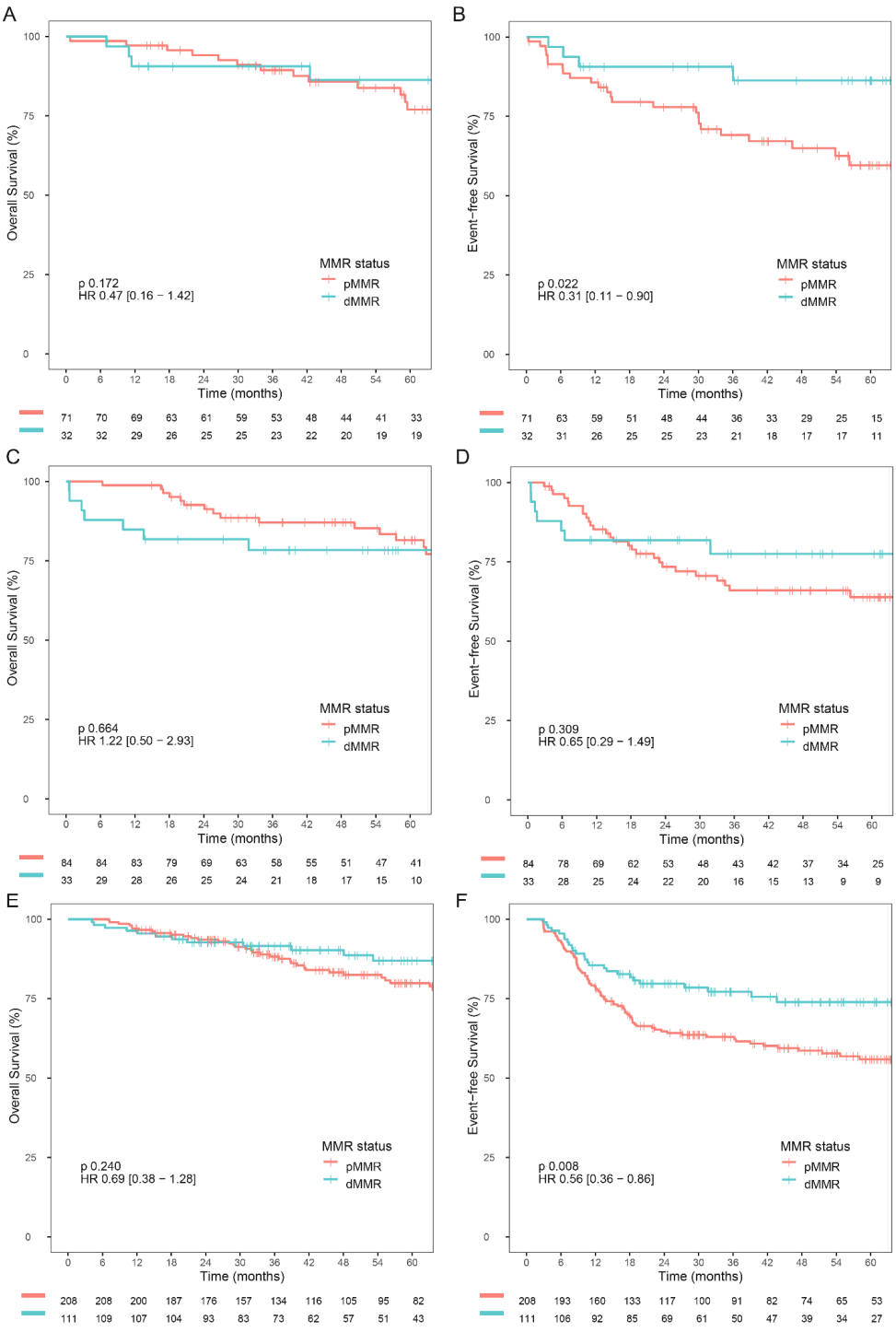
Supplementary Figure 1: Kaplan–Meier survival analyses according to MMR status in the matched cohort. A) OS for stage II disease; B) OS for stage III disease; C) EFS for stage II disease; D) EFS for stage III disease.
CI, confidence interval; dMMR, mismatch repair deficiency; EFS, event-free survival; HR, hazard ratio; MMR, mismatch repair; OS, overall survival; pMMR, proficient mismatch repair.



Supplementary Figure 2: Kaplan–Meier survival analyses of patients with a recurrence according to MMR status in the matched cohort.
CI, confidence interval; dMMR, mismatch repair deficiency; HR, hazard ratio; MMR, mismatch repair; pMMR, proficient mismatch repair.



Supplementary Figure 3: Kaplan–Meier survival analyses according to MMR status in the matched cohort. A) OS of patients treated with upfront surgery; B) EFS of patients treated with upfront surgery; C) OS of patients treated with neoadjuvant short-course radiotherapy; D) EFS of patients treated with neoadjuvant short-course radiotherapy; E) OS of patients treated with long-course neoadjuvant treatment; F) EFS of patients treated with long-course neoadjuvant treatment.
CI, confidence interval; dMMR, deficient mismatch repair; EFS, event-free survival; HR, hazard ratio; MMR, mismatch repair; OS, overall survival; pMMR, proficient mismatch repair.


A
Overall Survival (%)
p 0.172
HR 0.47 [0.16 − 1.42]
MMR status
pMMR
dMMR
Time (months)
71 70 69 63 61 59 53 48 44 41 33
32 32 29 26 25 25 23 22 20 19 19
B
Event−free Survival (%)
p 0.022
HR 0.31 [0.11 − 0.90]
MMR status
pMMR
dMMR
Time (months)
71 63 59 51 48 44 36 33 29 25 15
32 31 26 25 25 23 21 18 17 17 11
C
Overall Survival (%)
p 0.664
HR 1.22 [0.50 − 2.93]
MMR status
pMMR
dMMR
Time (months)
84 84 83 79 69 63 58 55 51 47 41
33 29 28 26 25 24 21 18 17 15 10
D
Event−free Survival (%)
p 0.309
HR 0.65 [0.29 − 1.49]
MMR status
pMMR
dMMR
Time (months)
84 78 69 62 53 48 43 42 37 34 25
33 28 25 24 22 20 16 15 13 9 9
E
Overall Survival (%)
p 0.240
HR 0.69 [0.38 − 1.28]
MMR status
pMMR
dMMR
Time (months)
208 208 200 187 176 157 134 116 105 95 82
111 109 107 104 93 83 73 62 57 51 43
F
Event−free Survival (%)
p 0.008
HR 0.56 [0.36 − 0.86]
MMR status
pMMR
dMMR
Time (months)
208 193 160 133 117 100 91 82 74 65 53
111 106 92 85 69 61 50 47 39 34 27

Supplementary Figure 4: Treatment responses of patients treated with chemoradiation according to MMR status in the matched cohort. A) Radiological responses after chemoradiation; B) Pathological responses after chemoradiation; C) Event-free survival of patients treated with neoadjuvant chemoradiation; D) Overall survival of patients treated with chemoradiation.
CI, confidence interval; dMMR, deficient mismatch repair; HR, hazard ratio; MMR, mismatch repair; pCR, pathological complete response; pMMR, proficient mismatch repair.

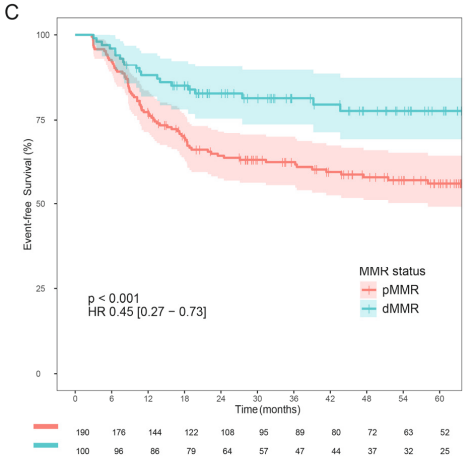
A

	pMMR (n=170)	dMMR (n=76)	p-value
Radiological response after neo-adjuvant treatment			
Complete response	14 (8.2%)	17 (22.4%)	0.023
Partial response	135 (79.4%)	53 (69.7%)	
Mixed response	1 (0.6%)	1 (1.3%)	
Stable disease	8 (4.7%)	2 (2.6%)	
Progressive disease	9 (5.3%)	1 (1.3%)	
Unknown	3	2	

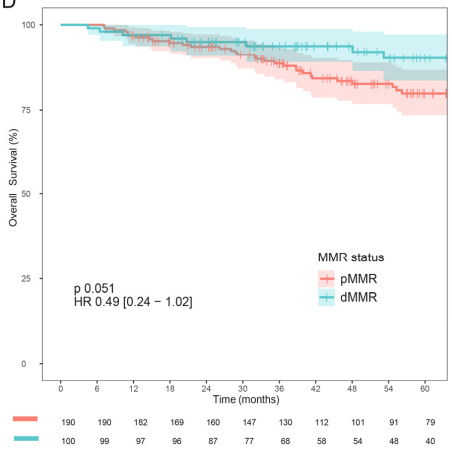
B

	pMMR (n=152)	dMMR (n=72)	p-value
Tumor regression grade (Mandard score)			
Full regression	20 (13.2%)	23 (31.9%)	0.006
Moderate regression	110 (72.4%)	43 (59.7%)	
Minor regression	3 (2.0%)	1 (1.4%)	
No signs of regression	7 (4.6%)	4 (5.6%)	
Missing	12	1	
Pathological complete response			
pCR	18 (11.8%)	23 (31.9%)	<0.001
No pCR	134 (88.2%)	40 (68.1%)	

C



D



Supplementary Table 1: Description of different secondary outcomes.
CT, computed tomography; MRI, magnetic resonance imaging; TEM, transanal endoscopic microsurgery; TME, total mesorectal excision.

Outcome	Description
Radiological response after neoadjuvant treatment	Complete response, partial response, mixed response, stable disease, or progressive disease according to the conclusion in the radiology report available in the electronic health record
Pathological response after neoadjuvant treatment	Response according to the pathology report available in the electronic health record
Organ preservation rate	Defined percentage of patients remaining TME-free after 12 months and having a sustained clinical complete response
Watch-and-wait strategy	Patients selected in the watch-and-wait group received neoadjuvant treatment only, no resection, and had either 1. a radiological complete response measurement, 2. ycT0N0M0, 3. no resection due to low tumor load as stated by physician, or 4. a TEM and full regression afterwards.
Chance of organ preservation	Defined percentage of patients remaining TME-free after 12 and 24 months or receiving resection but showing a pathological complete response
Clinical complete response at 12 months	For patients undergoing a watch-and-wait strategy (no resection), no signs of disease at or before 12 months both locally based on MRI and endoscopy and no signs of metastases on CT scan
Permanent stoma rate	Defined as a stoma present 12 months after placement

Supplementary Table 2: Patient, tumor characteristics, and treatment patterns of the matched cohort according to MMR status.
APR, abdominal peritoneal resection; ASA, American society of anesthesiologists; BRAF, B-RAF proto-oncogene serine/threonine kinase; c, clinical; dMMR, deficient mismatch repair; IQR, interquartile range; LAR, low anterior resection; N, node; Nx, unknown N stage; pMMR, proficient mismatch repair; RAS, rat sarcoma; T, tumor; Tx, unknown T stage; WHO, World Health Organization.

	dMMR (n=176)	pMMR (n=363)	p-value
Age (years)			0.080
Median (IQR)	57.5 (18.3)	59.0 (17.0)	
Sex			0.418
Male	99 (56.3%)	219 (60.3%)	
Female	77 (43.8%)	144 (39.7%)	
Year of diagnosis			1
2015	12 (6.8%)	25 (6.9%)	
2016	27 (15.3%)	56 (15.4%)	
2017	29 (16.5%)	64 (17.6%)	
2018	21 (11.9%)	44 (12.1%)	
2019	20 (11.4%)	40 (11.0%)	
2020	22 (12.5%)	44 (12.1%)	
2021	29 (16.5%)	56 (15.4%)	
2022	16 (9.1%)	34 (9.4%)	
WHO performance status			0.142
0	80 (66.1%)	199 (71.8%)	
1	38 (31.4%)	66 (23.8%)	
2	3 (2.5%)	11 (4.0%)	
3	0 (0%)	1 (0.4%)	
Missing	55	86	
ASA score			0.241
Class I	33 (20.8%)	88 (25.8%)	
Class II	100 (62.9%)	213 (62.5%)	
Class III	24 (15.1%)	37 (10.9%)	
Class IV	2 (1.3%)	3 (0.9%)	
Missing	17	22	
cT stage			0.723
T1	1 (0.6%)	0 (0%)	
T2	18 (10.2%)	36 (9.9%)	
T3	113 (64.2%)	239 (65.8%)	
T4	39 (22.2%)	75 (20.7%)	
Tx	5 (2.8%)	13 (3.6%)	
cN stage			0.993
N0	42 (23.9%)	89 (24.5%)	
N1	58 (33.0%)	120 (33.1%)	
N2	74 (42.0%)	149 (41.0%)	
Nx	2 (1.1%)	5 (1.4%)	
BRAF mutation			0.014
Wild type	23 (79.3%)	51 (98.1%)	
Mutant	6 (20.7%)	1 (1.9%)	
Missing	147	311	

	dMMR (n=176)	pMMR (n=363)	p-value
RAS mutation			0.114
Wild type	11 (64.7%)	25 (43.6%)	
Mutant	6 (35.3%)	29 (53.7%)	
Missing	159	309	
Differentiation grade			<0.001
Good	4 (2.5%)	5 (1.5%)	
Moderate	126 (78.8%)	317 (92.4%)	
Poor	30 (18.8%)	21 (6.1%)	
Missing	16	20	
Histology			0.010
Adeno	153 (86.9%)	341 (93.9%)	
Mucinous	17 (9.7%)	20 (5.5%)	
Signet ring cell	4 (2.3%)	2 (0.6%)	
Medullary type	2 (1.1%)	0 (0%)	
Distance to anal verge			0.069
0–5 cm	91 (54.8%)	184 (52.1%)	
5–10 cm	53 (31.9%)	119 (33.7%)	
10–15 cm	22 (13.3%)	40 (11.3%)	
>15 cm	0 (0%)	10 (2.8%)	
Missing	10	10	
Treatment type			0.181
Neoadjuvant treatment only	34 (19.3%)	48 (13.2%)	
Neoadjuvant treatment and resection	110 (62.5%)	244 (67.2%)	
No neoadjuvant treatment (upfront resection)	32 (18.2%)	71 (19.6%)	
Resection of primary tumor			0.085
No	34 (19.3%)	48 (13.2%)	
Yes	142 (80.7%)	315 (86.8%)	
Type of resection			1
Other than APR (e.g. LAR)	106 (60.2%)	235 (64.7%)	
APR	36 (20.5%)	80 (22.0%)	
Stoma			1
Yes	113 (64.2%)	233 (64.2%)	
No	63 (35.8%)	130 (35.8%)	
Neoadjuvant treatment type			0.481
Chemoradiation (± systemic)	100 (56.8%)	190 (52.3%)	
Radiotherapy only	34 (19.3%)	87 (24.0%)	
Radiotherapy + systemic	8 (4.5%)	14 (3.9%)	
Chemotherapy only	1 (0.6%)	1 (0.3%)	
Targeted therapy	1 (0.6%)	0 (0%)	
No neoadjuvant treatment	32 (18.2%)	71 (19.6%)	
Adjuvant treatment			0.990
No	166 (94.3%)	344 (94.8%)	
Yes	10 (5.7%)	19 (5.2%)	

Supplementary Table 3. Recurrence patterns and metastatic sites according to MMR status of patients in the matched cohort.
dMMR, deficient mismatch repair; pMMR, proficient mismatch repair.

	dMMR (n=176)	pMMR (n=363)
First recurrence type		
No recurrence	150 (85.2%)	241 (66.4%)
Locoregional recurrence	8 (4.5%)	22 (6.1%)
Metastatic recurrence	16 (9.1%)	87 (24.0%)
Both locoregional failure and metastasis	1 (0.6%)	12 (3.3%)
Unknown recurrence type	1 (0.6%)	1 (0.3%)
Metastatic sites of first recurrence		
Liver	5 (29.4%)	46 (46.5%)
Pulmonary	3 (17.6%)	29 (29.3%)
Bone	2 (11.8%)	3 (3.0%)
Spleen	0 (0%)	1 (1.0%)
Skin	0 (0%)	1 (1.0%)
Peritoneal	4 (23.5%)	7 (7.1%)
Other	0 (0%)	4 (4.0%)
Brain	0 (0%)	1 (1.0%)
Adrenal gland	1 (5.9%)	0 (0%)
Lymph nodes	2 (11.8%)	7 (7.1%)
Amount of metastatic sites (of all first metastatic recurrences)		
1	14 (82.4)	70 (70.7%)
2	3 (17.6)	23 (23.2%)
3	0 (0%)	5 (5.1%)
4	0 (0%)	1 (1.0%)
Liver, lung, or peritoneal only (of first metastatic recurrences)		
Other	7 (41.2%)	41 (41.4%)
Liver only	4 (23.5%)	32 (32.3%)
Lung only	3 (17.6%)	21 (21.2%)
Peritoneum only	3 (17.6%)	5 (5.1%)

Part II

Biomarkers for recurrence risk and response to adjuvant CAPOX in CC

4

Circulating tumor DNA (ctDNA) in patients with stage III colon cancer: the multicenter prospective PROVENC3 study

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*Equal contribution

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Abstract

Introduction

Circulating tumor DNA (ctDNA) is a promising biomarker to guide clinical decision making. The aim of this study was to investigate the prognostic value of post-surgery ctDNA in patients with stage III colon cancer who received adjuvant chemotherapy (ACT).

Methods

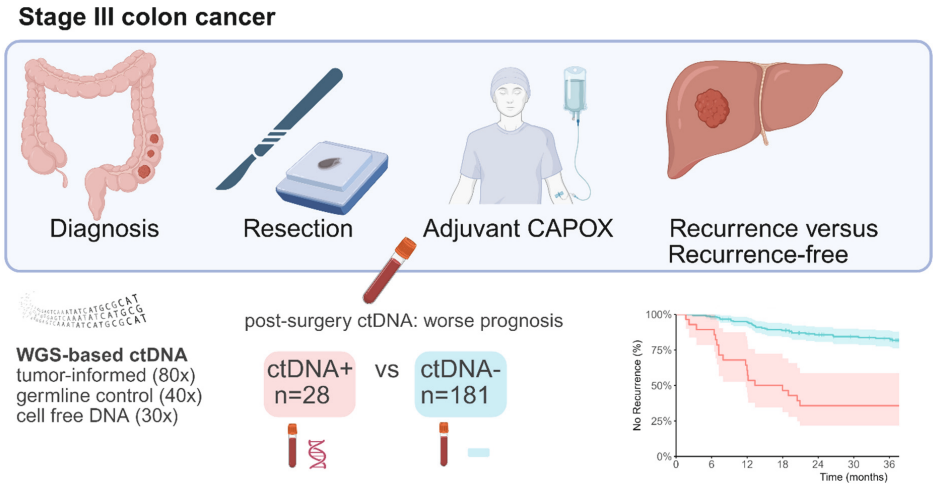
PROVENC3 was a multicenter prospective study of patients who underwent resection of pathological stage III colon cancer. Blood samples were collected at a median of 13 days (interquartile range 4-20 days) after resection. Presence of minimal residual disease was determined using Labcorp® Plasma Detect™, a novel tumor-informed whole genome sequencing (WGS) ctDNA test. Primary endpoint was 3-year (time to recurrence). ctDNA status was further combined with pathological risk status to investigate combined prognostic value.

Results

Median follow-up of 209 patients included was 40 months. 28 patients had detectable ctDNA post-surgery (13%). Post-surgery ctDNA-positive patients had a worse TTR compared to ctDNA-negative patients (hazard ratio (HR) 6.2 [95%CI 3.4-11.2], p<0.001). Of all ctDNA-positive patients, 36% did not develop recurrences during 3-year follow-up. Detectable ctDNA after ACT was associated with worse TTR (HR 7.9 [3.9-15.9]; p<0.001). ctDNA status combined with pathological risk classification resulted in a 3-year recurrence risk varying from 82% for pathological high-risk (pT4/N2) ctDNA-positive patients to 7% for pathological low-risk (pT1-3N1) ctDNA-negative patients (HR 28.5 [10.5-77.2], p<0.001).

Conclusion

Post-surgery ctDNA detection using a tumor-informed WGS test improves stratification of prognosis in stage III colon cancer and may help to personalize adjuvant treatment.


Stage III colon cancer
Diagnosis
Resection
Adjuvant CAPOX
Recurrence versus
Recurrence-free
WGS-based ctDNA
tumor-informed (80x)
germline control (40x)
cell free DNA (30x)
post-surgery ctDNA: worse prognosis
ctDNA+
n=28
vs
ctDNA-
n=181
No Recurrence (%)
100%
75%
50%
25%
0%
0
6
12
18
24
30
36
Time (months)

Introduction

Surgery followed by adjuvant chemotherapy (ACT) is standard of care for non-metastatic patients with node-positive (stage III) colon cancer.[1] Approximately 55% of patients are however cured by surgery alone, and 30% experience a recurrence despite ACT.[2,3] As a consequence, only 15-20% of patients benefit from ACT, whilst all patients are exposed to the risk of developing side effects. Therefore, there is an urgent unmet need to identify those patients who are truly at risk of recurrence after surgery and could benefit from ACT and those who are at a low risk of developing recurrence and could avoid ACT.

Detection of circulating tumor DNA (ctDNA) in minimally invasive blood liquid biopsies is a clinically applicable technology to retrieve information about solid tumors, even when tissue biopsies cannot be obtained. One putative clinical application is the post-surgery detection of ctDNA in cell-free plasma as a biomarker for minimal residual disease (MRD).[4] Recent studies have demonstrated that post-surgery ctDNA is a strong prognostic biomarker for disease recurrence in stage II and III colon cancer. This could enable identification of patients at high risk of developing recurrence and guide clinical decisions.[5-9] However, MRD detection after resection of the primary tumor is technically demanding due to extremely low levels of ctDNA.[10] These challenges have been primarily mitigated through tumor-informed personalized ctDNA approaches to maximize sensitivity and specificity, which often require patient-specific bespoke panels to be developed. This introduces several operational and technical complexities into the assay workflow, which prolongs turnaround times for the post-surgery landmark test result and complicates incorporation of ctDNA-based MRD testing into specific clinical settings. Latest developments in the field have shown that tumor-informed whole genome sequencing (WGS) approaches hold promise for MRD testing, given the ability to track thousands of tumor-specific mutations without the need for personalized assay design, manufacture, and quality control testing for each patient, and can result in landmark testing turnaround times of <14 days versus four to six weeks for bespoke panel-based approaches based on a more limited number of alterations.[11-14] The first study evaluating WGS-based ctDNA detection has been recently published[15], yet the clinical performance of these methods remains to be fully established in independent prospective clinical cohorts.

The prospective colorectal cancer (PLCRC) cohort is a real-world, nationwide cohort study of patients with colorectal cancer (CRC) in the Netherlands.[16] PROVENC3 was a prospective observational substudy of PLCRC, consisting of a large, unselected group of patients treated according to the standard of care. The aim of the study was to determine the prognostic value of post-surgery ctDNA status in patients with stage III colon cancer treated with ACT.

Methods

Patients

This was a multicenter prospective study of patients with stage III colon cancer who underwent ACT. Patients diagnosed with CRC, 18 years or older and mentally competent, were prospectively recruited in the Netherlands for participation in the ongoing PLCRC (NCT02070146). Informed consent for the collection of long-term clinical and survival data is mandatory for participation in PLCRC. Subsequently, patients are given the option to consent for: 1) filling out questionnaires on health-related quality of life, functional outcomes and workability; 2) biobanking of tumor and normal tissue; 3) collection of blood samples; and 4) to be offered studies conducted within the infrastructure of the cohort. Clinical data was collected by the Netherlands Comprehensive Cancer Organization (IKNL) in the Netherlands Cancer Registry (NCR). Treatment-naïve non-metastatic CRC patients who gave informed consent for PLCRC and for additional blood sampling were included in the observational PLCRC substudy MEDOCC (Molecular Early Detection of Colon Cancer).

Patients with pathological stage III colon cancer, who started ACT after surgery and of whom post-surgery blood was available, were included in the PLCRC-MEDOCC substudy PROVENC3 (listed under PLCRC, NCT02070146) in 26 hospitals from 2016 to 2021. As a substudy within a real-world cohort, post-surgery blood collection was performed as part of routinely scheduled care blood withdrawals during standard of care visits. Patients with blood collected less than 3 days post-surgery were excluded from the study due to potential increased risk of primarily false negative results due to increased cell-free DNA levels due to surgical-induced trauma. After curation of clinical data, two patients were included whose blood had been collected two days after resection of the primary tumor. Clinical data was collected via the NCR and through site visits by the PLCRC study team.

The PLCRC study was performed in accordance with the Declaration of Helsinki and approved by the Medical Ethical Committee Utrecht (NL47888.041.14). All patients signed written informed consent for study participation and collection of blood and tissue samples for translational research. The PLCRC-substudy PROVENC3 was approved by the institutional review board (Institutional Review Board, IRB) of the Netherlands Cancer Institute, Amsterdam, the Netherlands (protocol CFMPB472).

Sample collection and processing

Formalin-fixed paraffin-embedded (FFPE) tumor blocks were requested through the Dutch Nationwide Pathology Databank (Palga).[17] Hematoxylin and eosin (H&E) stained slides were evaluated by a study-specific pathologist, and the tumor area was outlined for macro-dissection and DNA was isolated from that region (see Supplementary Methods).

Two blood tubes (10 ml each) were collected pre-surgery (single time point), post-surgery (single time point before the start of adjuvant chemotherapy), after completion of ACT and at 12, 18, 24 and 36 months. Blood was collected using a cell-stabilizing BCT tube (Streck, La Vista, USA) in the participating hospitals and shipped to the Netherlands Cancer Institute. DNA was isolated from white blood cells (WBC) and from cell-free plasma (see Supplementary Methods).

WGS-based tumor-informed plasma ctDNA detection

Labcorp® Plasma Detect™ is a novel tumor-informed WGS-based plasma ctDNA test for detection of MRD after curative intent intervention, including surgery or ACT. In brief, WGS data of tumor DNA (targeting 80x coverage) is compared to WGS data of WBC-derived germline DNA WGS data (targeting 40x coverage) to detect thousands of tumor-specific mutations that are unique to each patient. Next, WGS data obtained from 10 ng plasma-derived cell-free DNA (targeting 30x coverage) were used to search for the presence of a subset of these tumor-specific mutations in blood draws, using a proprietary and independently validated bioinformatics pipeline. Labcorp Plasma Detect then provides a result for ctDNA status together with an estimated tumor fraction for the samples considered ctDNA positive, referred to as Aggregate ctDNA variant allele fraction (VAF). A detailed description of the Labcorp Plasma Detect ctDNA test and its analytical test performance is provided in the Supplementary Materials.

Sample size calculation

In this prospective observational study, we aimed to evaluate the percentage of post-surgery ctDNA-positive patients using a novel tumor-informed whole genome sequencing plasma ctDNA test in a real-world cohort, and we aimed to evaluate the proportion of post-surgery ctDNA-positive patients who recur at 3 years in comparison to this proportion among ctDNA-negative patients. Because the latter question required the larger number of patients, the rationale for the sample size calculation was based on this question. We assumed 15% ctDNA-positivity and an overall recurrence rate at 3 years of 32%. To detect a difference in 3-year recurrence rate of at least 25% (28% in ctDNA-negative and 53% in ctDNA-positive patients), 189 patients would be required using a two-sided log-rank test with a significance level of 5% and power of 80%. To account for 10% drop-out, we started with a total cohort of 210 patients.

Statistical analysis

The prognostic value of ctDNA status was evaluated using time to recurrence (TTR) as the outcome measure. For TTR analyses, only recurrences were considered as events and patients without a recurrence were censored at the last visit to the treating physician, regardless of survival status.[18] Follow-up was censored at year 3, reporting 3-year recurrence risk (RR). For univariable TTR analyses, the Kaplan Meier estimator and fitted Cox regression models were used. The pathological variables evaluated in the univariable models were selected based on clinical relevance: Pathological risk status (Low risk=T1-3N1, High risk=T4 and/or N2), T status based on the pathology report (T1-3, T4), N status based on the pathology report (N1, N2), and microsatellite instability (MSI) status determined by next-generation sequencing of the primary tumor (stable, instable). The change in the hazard ratios (HRs), reported with 95% confidence interval (CI), was also evaluated in univariable models after adding ctDNA status to each of the pathological covariates (Pathological risk + ctDNA status; T status + ctDNA status; N status + ctDNA status; MSI status + ctDNA status).

Furthermore, to evaluate whether post-surgery ctDNA status had added and independent predictive value for recurrence in addition to multiple pathological variables, several Cox regression models were fitted. First, the added value of ctDNA status was determined by fitting multivariable models combining the pathological risk factors and ctDNA status and performing likelihood ratio test among them (LRT 1:

Pathological risk versus Pathological risk + ctDNA status; LRT 2: Pathological risk + MSI status versus Pathological risk + MSI status + ctDNA status; LRT 3: T status + N status versus T status + N status + ctDNA status; LRT 4: T status + N status + MSI status versus T status + N status + MSI status + ctDNA status). Second, we evaluated the independent predictive value of each variable in the model by calculating the HRs of each variable independently in the two best models resulting from the LRT (Model 1: Pathological risk + MSI status + ctDNA status; Model 2: T status + N status + MSI status + ctDNA status). All statistical and survival analysis were performed using R package *survival* for survival (https://cran.r-project.org/web/packages/survival/index.html, R v4.4.1). Differences in baseline characteristics for the post-surgery ctDNA-positive versus post-surgery ctDNA-negative groups were analyzed using Fisher's exact test for categorical variables, and Mann-Whitney U test for continuous variables.

Availability of data and materials

The main data associated with this study are provided in the main text, Supplementary Materials and Methods and Supplementary Tables. Raw WGS data are deposited in EGAS50000000804 and subject to controlled access via a Data Access Committee and Data Transfer Agreement, conforming to informed consent and GDPR regulations.

Results

Study population

Two-hundred and nine patients with stage III colon cancer who were treated with ACT were included in the PROVENC3 study (Supplementary Table 1). Of these, 188 (90.0%) patients received 3 months of capecitabine + oxaliplatin (CAPOX) as adjuvant treatment. The other patients were treated with 6 months FOLFOX (4.8%) or 6 months capecitabine monotherapy (5.2%). Post-surgery blood samples were obtained and analyzed for all 209 patients. In addition, pre-surgery and post-adjuvant chemotherapy (post-ACT) blood samples were analyzed for 148 and 171 patients, respectively (Supplementary Figure 1). Median follow-up was 40 months (interquartile range (IQR) 21-57 months), and 47 (22.5%) patients developed a recurrence within three years post-surgery. Median follow-up for patients not developing a recurrence was 45 months (range 38-74 months).

Analytical performance of the ctDNA detection test

Post-surgery detection of ctDNA indicates presence of MRD, however application of a highly sensitive tumor-informed plasma ctDNA test is essential to enable detection of the extremely low levels of ctDNA in this clinical setting. The novel Labcorp® Plasma Detect™ test was demonstrated to have a high analytical sensitivity, with a 95% limit of detection of 0.005% tumor content and a 50% limit of detection of 0.001% tumor content through integrated WGS analyses of patient-matched FFPE tumor tissue DNA, WBC-derived normal germline DNA and plasma cfDNA (Supplementary Results; Supplementary Figure 2; Supplementary Tables 2-4). Moreover, among 148 patients for whom pre-surgery blood samples were available, ctDNA could be detected in 135 patients (91.2%), underscoring the high ctDNA test clinical sensitivity.

Post-surgery detection of ctDNA

Post-surgery blood was available for 241 patients. Thirty-two samples could not be analyzed due to insufficient tumor tissue (n=3), plasma DNA (n=5) or quality control (QC) failure (n=19) (Supplementary Figure 1b). Post-surgery ctDNA status was determined in 209 patients at a landmark time point with a single blood draw at a median of 13 days after surgery (IQR 4-20 days; Supplementary Table 5). Twenty-eight out of 209 (13.4%) patients were ctDNA-positive and 181 (86.6%) were ctDNA-negative after surgery. The post-surgery median aggregate ctDNA VAF was 0.035% (range 0.01%-3.13%) (Supplementary Table 5). Of the evaluated baseline pathological features, only irradical resection was associated with post-surgery ctDNA-positivity (Supplementary Table 1). Pre-surgery ctDNA status was not related to post-surgery ctDNA status (Fisher p=0.741).

Surgical trauma leads to increased levels of cfDNA in blood during the first week post-surgery, which may obscure and confound ctDNA detection and therefore lead to false negative results.[19] Relative to the day of surgery, 30.6% of the post-surgery blood samples were collected before day 7, 21% between days 7-13, 26.8% between days 14-20 and 21.5% from day 21 onward. In the present study, using the sensitive Labcorp Plasma Detect test, there was no significant difference in post-surgery ctDNA detection when blood was collected within one week post-surgery or later (Supplementary Figure 3).

Prognostic value of post-surgery ctDNA status

Post-surgery ctDNA-positive patients had a worse TTR compared to ctDNA-negative patients (HR 6.2 [95%CI 3.4-11.2]; p<0.001; Figure 1A-B). Among the 28 post-surgery ctDNA-positive patients, 10 (35.7%) remained recurrence-free during 36 months follow-up, suggesting they may have benefited from ACT (Figure 1A).

When assessing pathological risk, 127 (60.8%) patients had low-risk (pT1-3N1) and 82 patients (39.2%) had high-risk (pT4/pN2) tumors. In univariable analysis, pathological high-risk was associated with recurrence (HR 3.5 [1.9-6.5]; p<0.001) (Figure 1C-D; Supplementary Table 6). The prognostic value of the combination of pathological risk and post-surgery ctDNA status was evaluated in multivariable analysis. RR of pathological high-risk patients was further increased when patients were ctDNA-positive, while RR of pathological low-risk patients was further decreased when patients were ctDNA-negative. Consequently, there is a profound TTR difference between pathological high-risk ctDNA-positive patients (n=11) and pathological low-risk ctDNA-negative patients (n=110) (3-year RR 81.8% versus 6.7%, HR 28.5 [10.5-77.2]; p<0.001) (Figure 1E-F; Supplementary Table 7; Supplementary Figure 4). ctDNA status improved the multivariable Cox regression model including pathological risk and MSI status (LRT p<0.001) (Supplementary Table 8, LRT1). ctDNA status was the strongest independent predictor of recurrence (HR 6.8) in a model that also included pathological risk (HR 4.0) and MSI status (HR 0.7; ns) (Supplementary Table 9). In addition, post-surgery ctDNA status remained the strongest predictor of recurrence in multivariable models that included T, N and MSI status as independent risk factors (Supplementary Tables 6-9).

Among the 47 out of 209 (22.5%) patients who experienced a recurrence within 3 years, post-surgery ctDNA-positive patients had a shorter time to recurrence than ctDNA-negative patients (Wilcoxon p=0.030) (Supplementary Figure 5A). Because ctDNA shedding can be affected by the metastatic site, the association between recurrence location and post-surgery ctDNA status was examined. Eight out of 11 (73%) patients with liver-limited metastases were ctDNA-positive post-surgery, while none out of five patients with peritoneal metastases were ctDNA-positive (Supplementary Figure 5B).

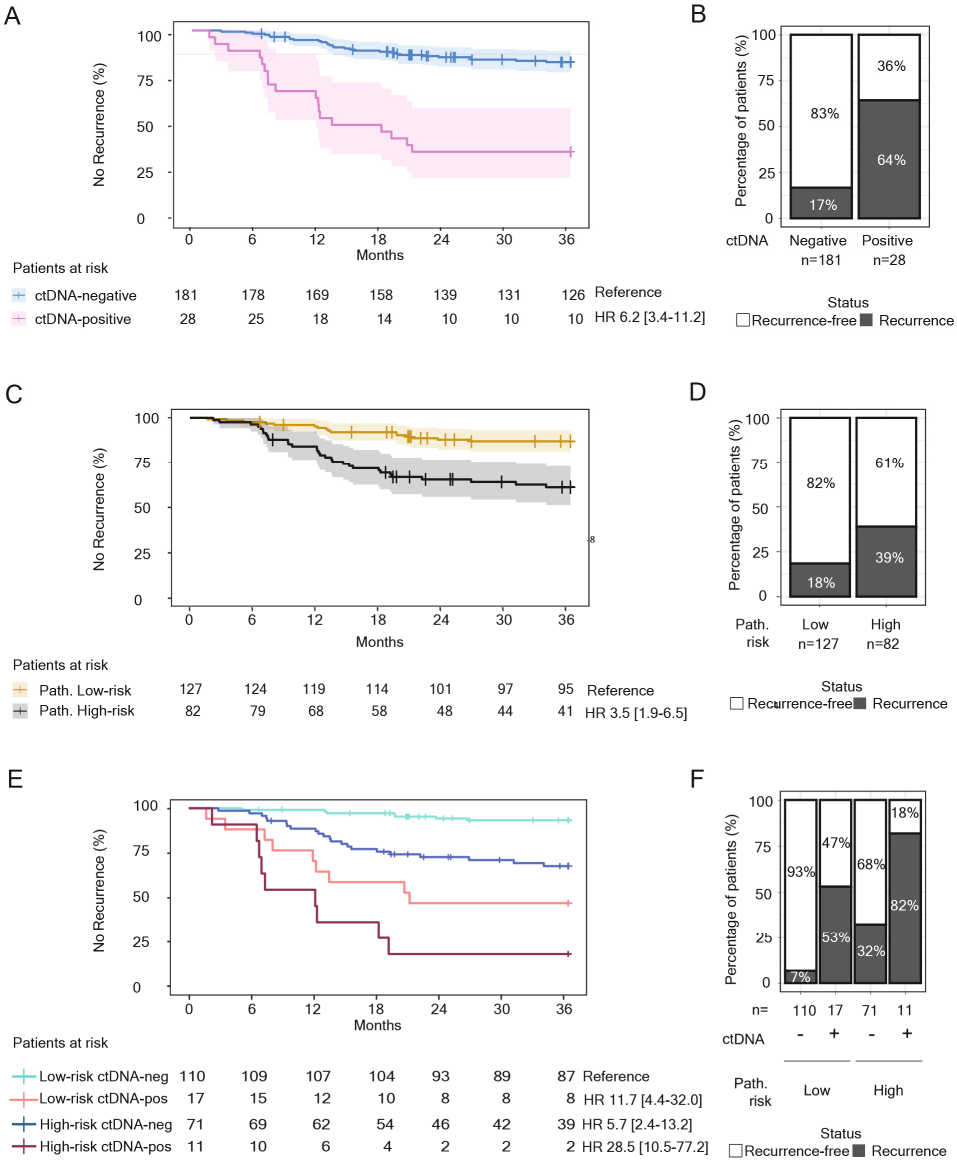


Figure 1: Detection of ctDNA post-surgery is independently associated with recurrence at three years in ACT-treated stage III colon cancer. A) Time to recurrence stratified by post-surgery ctDNA status and B) proportion of patients with recurrence after three years; C) Time to recurrence stratified by pathological risk and D) proportion of patients with recurrence after three years; E) Time to recurrence stratified by pathological risk and ctDNA status and F) proportion of patients with recurrence after three years. *ACT, adjuvant chemotherapy; ctDNA, circulating tumor DNA; ctDNA-pos, ctDNA-positive; ctDNA-neg, ctDNA-negative; HR, hazard ratio; n, number; path. pathological.*

Prognostic value of post-ACT ctDNA status

Following adjuvant treatment, post-ACT ctDNA analysis could be investigated in 171 of the 209 patients (Supplementary Figure 6A and 6B). Twenty-four patients were ctDNA-positive after ACT, which was associated with a poor TTR compared to ctDNA-negative patients after ACT (HR 7.9 [3.9-15.9]; p<0.001) (Figure 2A and 2B). Dynamic changes in ctDNA status ('negative' or 'positive') before and after ACT were evaluated by combining post-surgery and post-ACT ctDNA results (Figure 2C). All patients in the ctDNA positive-positive group (n=7) experienced disease recurrence, indicating that treatment with ACT did not succeed to eliminate disease. In contrast, the 3-year RR of the ctDNA negative-negative group (n=137) was 10.6%. Ten of the 17 patients (58.8%) who were ctDNA-positive post-surgery became ctDNA-negative post-ACT (positive-negative) and had a risk of recurrence which was not different from the ctDNA negative-negative group (Cox regression, p=0.074), suggesting benefit from ACT. The ctDNA negative-positive group had an intermediate risk of recurrence, with an increased risk of disease recurrence when compared to the ctDNA negative-negative group (Cox regression, p<0.001). Notably, ctDNA levels in the majority of patients in the ctDNA positive-negative and negative-positive groups were close to the limit of detection of the ctDNA test and lower than the ctDNA levels observed in the ctDNA positive-positive patients (Figure 2D). The highest ctDNA levels (>0.10%) corresponded to patients experiencing a recurrence.

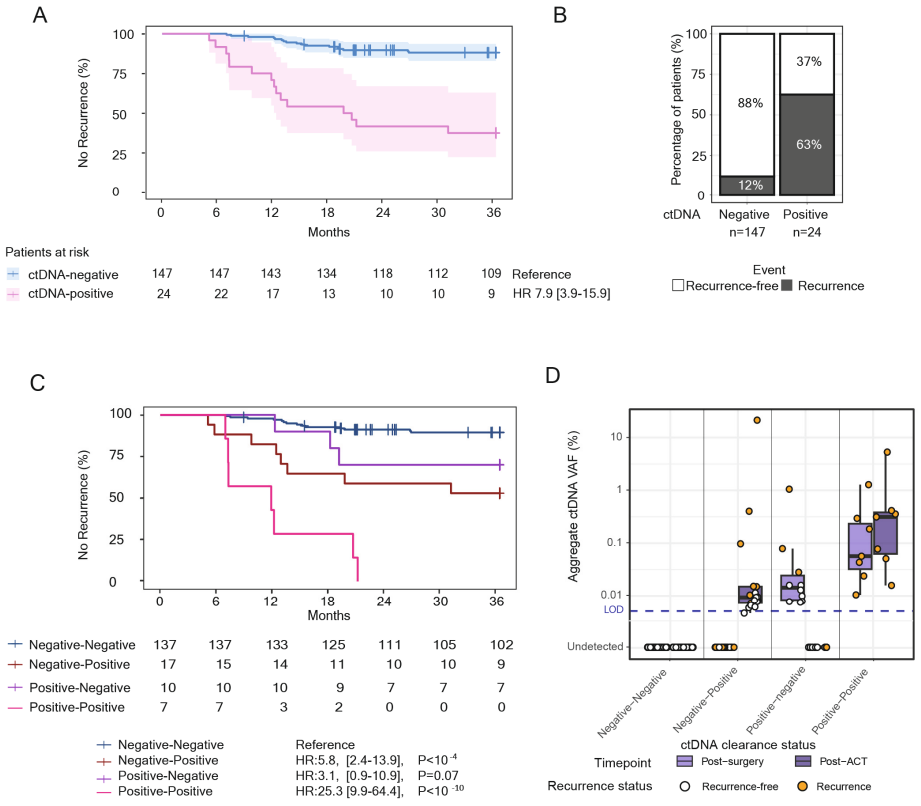


Figure 2: Evaluation of prognostic value of post-ACT ctDNA detection. A) Time to recurrence stratified by post-ACT ctDNA status and B) proportion of patients with recurrence after three years; C) Time to recurrence stratified by ctDNA dynamics based on post-surgery and post-ACT ctDNA status; D) Aggregate ctDNA VAF on a logarithmic scale for each combination of post-surgery - post-ACT ctDNA test results.
ACT, adjuvant chemotherapy; ctDNA, circulating tumor DNA; HR, hazard ratio; VAF, variant allele frequency.

Post-ACT ctDNA surveillance for early detection of recurrences

Longitudinal ctDNA analyses were performed for the 13 patients who experienced a recurrence and had more than one post-ACT blood collection time point available (Figure 3). Three out of these 13 patients were ctDNA-positive immediately post-surgery. One of these three patients remained ctDNA-positive at all longitudinal time points evaluated while in the two other patients ctDNA was cleared in the first blood sample collected after completion of adjuvant chemotherapy and became detectable again at the second time point evaluated after ACT. Seven of the 10 post-surgery ctDNA-negative patients became ctDNA-positive during longitudinal follow-up. Three out of the 13 patients were ctDNA-negative for all the longitudinal samples evaluated from surgery of whom two were also ctDNA-negative pre-surgery. These three patients developed metastases in peritoneum, local, and intra-abdominal lymph nodes. Taken together, these data demonstrate a clinical sensitivity for recurrence detection prior to clinical recurrence of 76.9% (10/13) in the surveillance setting, with molecular recurrence detected for the first time (i.e. first ctDNA-positive sample during follow-up) at a median of 7.6 months before recurrence (min=33 days, max=412).

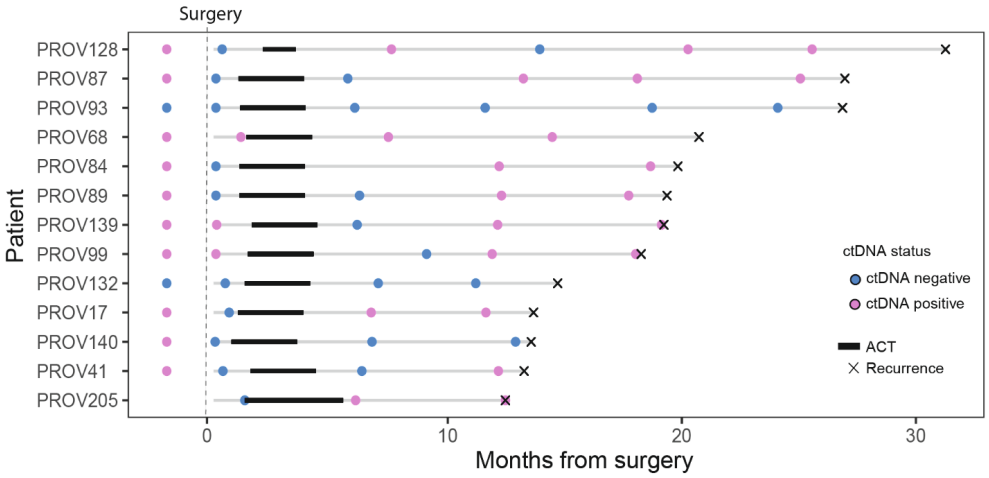


Figure 3: Swimmer plot of all plasma samples evaluated for the 13 patients who experienced a recurrence and for whom more than one post-ACT sample was available.
ACT, adjuvant chemotherapy; ctDNA, circulating tumor DNA.

Discussion

The PROVENC3 study demonstrated that the risk of recurrence for patients with stage III colon cancer can be predicted performing post-surgery ctDNA testing, using a highly sensitive tumor-informed WGS ctDNA test. The prognostic value is further increased by combining post-surgery ctDNA status with pathological risk, which reveals a large subgroup (more than 50%) of low-risk ctDNA-negative patients who had a very low 3-year disease recurrence risk of 6.7%.

This study described a novel tumor-informed WGS ctDNA test with a robust analytical and clinical sensitivity, illustrated by the high percentage of patients with detectable ctDNA pre-surgery (91%), e.g. compared to a recently published WGS-based ctDNA detection method in a similar cohort of stage III CRC patients (84% sensitivity pre-surgery)[20] and to previously published next generation sequencing-based methods[21]. Nevertheless, 9% of cancers remained undetected pre-surgery, which could be due to pre-analytical or analytical factors, but also to variability in biological characteristics among primary tumors. Such variability in ctDNA-shedding properties appears more evident when evaluating the different location of metastatic lesions. For example, none of the patients who developed peritoneum-only metastases within three years had detectable ctDNA immediately post-surgery in this study. This observation is in agreement with reports in literature indicating that peritoneal metastases are difficult to detect by ctDNA-testing.[22,23] Therefore, while ctDNA assays are further developed with increasing analytical sensitivity, it remains important to identify the molecular and physiological features that define a high or low likelihood of detectable levels of ctDNA in blood, as ctDNA-based strategies may not be suited for RR stratification of the subset of “low shedding cancers”.

The clinical sensitivity of ctDNA to detect disease recurrence in the PROVENC3 study, both at the landmark post-surgery time point and longitudinally, is consistent with recent data indicating that ctDNA is detectable typically six to 10 months prior to clinically overt recurrence.[8] Post-surgery ctDNA testing holds promise to tailor ACT, to guide adjuvant escalation decisions by identifying the patients at (very) high risk of developing a recurrence, either at the MRD timepoint or after ACT. On the other hand, post-surgery ctDNA could guide de-escalation for a group of patients at very low risk

of recurrence. However, to further advance robust de-escalation strategies, more data regarding ctDNA status for patients who did not receive ACT is needed.

A considerable proportion of patients who were ctDNA-negative post-surgery developed recurrences, which indicates that ctDNA status alone is at this moment insufficient to routinely withhold ACT. Importantly, longitudinal WGS-based ctDNA testing detected ctDNA in most evaluated patients with recurrence, indicating that appropriate surveillance strategies need to be developed to improve disease management. ctDNA-based surveillance may provide a window of opportunity for earlier and potentially more effective intervention than is possible when the recurrence manifests clinically or on imaging. These early interventions may constitute local treatment with curative intent, including surgical resection or radiotherapy for localized recurrences, or systemic treatment in case of wider metastatic spread or even in the absence of macroscopic disease. However, the lead time of ctDNA testing to detect molecular recurrence versus CT scan, the need for confirmatory imaging procedures after a positive ctDNA test during surveillance, and the optimal frequency of longitudinal ctDNA testing remain to be systematically assessed. Moreover, longer follow-up in current and future studies is needed to evaluate whether intensified follow-up with early treatment leads to improved overall survival. Importantly, this will require a shift in mindset from the decades-old dogma of ‘one-size-fits-all’ treatment with ACT to more personalized ctDNA-guided adjuvant chemotherapy decision-making and interventions.

The clinical performance of the ctDNA test employed in the PROVENC3 study is similar to the results of tumor-informed bespoke panel-based approaches, including the GALAXY study in Japan and the DYNAMIC study in Australia (14.8% and 15.3% of patients harbored detectable ctDNA post-surgery, respectively).[5,9] Compared to the approaches used in those studies, the current study demonstrated that a WGS-based technique can achieve a similar clinical sensitivity to patient-specific bespoke panel-based methods, without the need to design, manufacture, and quality control custom panels for each patient. This results in a significantly shorter turnaround time (within 14 days versus four to six weeks from sample to report), which is especially relevant in the post-surgical setting, where ACT should preferably be initiated within eight weeks.

Each ctDNA detection assay has an analytical limit of detection, representing the lowest amount of ctDNA in blood that can be detected. At present, this analytical limit of detection needs to be improved to increase the clinical sensitivity for ctDNA detection and to detect a higher proportion of patients with occult disease. Technically, this may be achieved by including evaluation of additional tumor specific cell-free DNA features together with increases in the depth of sequencing and reductions in technical background error rates.[24,25] Clinically, when ctDNA levels are reported that are close to the limit of detection, the results may be influenced by stochastic variation in blood sampling and should be interpreted with caution. This is illustrated by our evaluation of the ctDNA dynamics in response to treatment with ACT: two patients who cleared ctDNA during adjuvant chemotherapy but experienced a recurrence had a an aggregate ctDNA VAF close to the limit of detection post-surgery and then became ctDNA-positive quickly during longitudinal surveillance. This phenomenon represents a limitation in the current state of ctDNA technologies to detect MRD, where variation in ctDNA status at very low ctDNA levels may occur due to a reduction or increase in overall tumor burden or may occur due to stochastic variation in ctDNA sampling.

Current stratification for adjuvant treatment in non-metastatic colon cancer is based on lymph node status. Compared to the current standard-of-care, post-surgery ctDNA testing offers a substantial improvement as it enables to identify patients with MRD who are highly likely to develop a recurrence if left untreated (in >95% of cases). At the same time, ctDNA-negative patients have a reduced risk of developing a recurrence. The main challenge in the MRD setting is that the ctDNA levels secreted by the few tumor cells still present in the body are very low, and even the most sensitive techniques will miss a number of cases (false-negatives). Importantly, the risk stratification presented here by the PROVENC3 study illustrates the added value of the combination of post-surgery ctDNA testing and pathological features, which allowed to identify a substantial group of patients with only 6.7% recurrence risk who might benefit from withholding ACT. Interventional studies are needed to demonstrate the clinical utility of the combination of ctDNA testing with pathological risk stratification to guide ACT decisions.

Recent studies have shown the potential benefit of the use of neoadjuvant therapy for non-metastatic colon cancer patients: chemotherapy for clinical stage T4b MSS[26], immunotherapy for MSI[27], and potentially a combination of both in T3-4, MSS, *RAS/RAF* wild type[28]. The implementation of neoadjuvant treatment for subgroups of stage III colon cancer patients could have implications for ctDNA testing strategies. Post-surgery detection of ctDNA in patients who are treated with neoadjuvant therapy will be even more challenging than its detection in treatment-naïve patients, due to the reduction in the number of cancer cells in micrometastatic lesions by the neoadjuvant treatment. Pre-surgery, ctDNA testing could be considered as a tool to stratify patients for neoadjuvant treatment or to evaluate the response to neoadjuvant treatment in a minimal-invasive manner. The clinical applicability and utility of ctDNA testing in the setting of neoadjuvant treatment would need to be carefully evaluated in prospective observational and interventional clinical trials.

Limitations of this study included lack of overall survival data in addition to TTR data, the focus on a single post-surgery landmark ctDNA timepoint, variation in timing of the post-surgery blood sample collection, heterogeneity in histopathological subtypes, QC failure in 19 out of 241 cases, and the fact that preoperative samples and post-ACT samples could not be collected for all patients.

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Supplementary material

Sample collection and processing

DNA was isolated from FFPE slides using the QIAGEN AllPrep DNA/RNA FFPE kit (QIAGEN, Hilden, Germany) and stored at -20°C or 4°C only for short term before shipment. DNA quality and quantity were measured on a Nanodrop One (Isogen, IJsselstein, The Netherlands) and on a Qubit 3.0 Fluorometer (Molecular Probes, Leiden, The Netherlands) with the use of the Qubit dsDNA High-Sensitivity Assay (Thermo Fisher Scientific, USA).

Cell-free plasma and white blood cells (WBC) were separated by centrifugation of the blood for 10 minutes at 1,700xg followed by 10 minutes at 20.000xg, then stored at -80°C until further processing. Cell-free DNA (cfDNA) was isolated from the available plasma using the QIAsymphony DSP Circulating DNA Kit (QIAGEN, Hilden, Germany) with a fixed elution volume of 60 µL. Genomic DNA was isolated from WBCs using the QIAsymphony DSP DNA Midi Kit (QIAGEN, Hilden, Germany) and 1 ml blood protocol. cfDNA and genomic DNA from WBCs was stored at -20°C until further processing. The Qubit dsDNA High-Sensitivity Assay (Thermo Fisher, Waltham, USA) was used to quantify DNA yield for next generation sequencing.

Labcorp plasma detect ctDNA test methodology

Labcorp Plasma Detect is a novel tumor-informed WGS-based plasma ctDNA test for detection of minimal residual disease after intervention with curative intent, including surgery or adjuvant chemotherapy. We here provide a detailed description of the Labcorp Plasma Detect ctDNA test methodology and associated analytical test performance.

Noncancerous donor plasma and commercial cell line cohorts

Noncancerous donor plasma samples were obtained under Institutional Review Board approval from Discovery Life Sciences (Alabama, USA). Human tumor and normal cells from previously characterized cell lines were obtained from ATCC (Virginia, USA) (COLO-829, HCC-1187, HCC-1143, HCC-1954) and SeraCare (Massachusetts, USA) (SeraSeq gDNA TMB-mix Score 26). Clinical samples collected under IRB-approved protocols from nine colorectal cancer patients (SU8790, SU8792, SU8794, SU8799, SU8810, SU8820, SU8830, SU8836, SU8837) and five head and neck patients (SU8804, SU8809, SU8821, SU8825, SU8842) were commercially procured. cfDNA was isolated from plasma using the Qiagen Circulating Nucleic Acid kit (Qiagen, Germany) and the concentration was assessed using the Qubit dsDNA High-Sensitivity Assay (Thermo Fisher, USA). Genomic DNA was isolated from cell line samples using the QIAamp DNA Blood Mini Kit (Qiagen, Germany) and the concentration assessed using the Qubit dsDNA Broad Range Assay (Thermo Fisher, USA).

WGS analysis of tissue-derived tumor DNA and WBC-derived germline DNA
White blood cell (WBC)-derived genomic DNA was quantified using the Qubit dsDNA Broad Range Assay (Thermo Fisher, USA) and up to 400 ng of DNA was sheared to a target fragment size of approximately 450 base pairs (bp) using Covaris focused ultrasonication (Covaris, USA). Additionally, genomic DNA derived from FFPE tumor tissue was repaired using the PreCR Repair Mix (New England Biolabs, USA). Whole-genome sequencing libraries were prepared from fragmented genomic DNA through end-repair, A-tailing, and adapter ligation with the KAPA HyperPrep reagent kit according to the manufacturer's protocol (Roche, USA). Subsequently, these libraries were amplified through 7 cycles of polymerase chain reaction (PCR), pooled, and sequenced with 150 bp paired-end reads using the Illumina NovaSeq6000 platform (Illumina, USA) to a target depth of 80x for tumor samples and 40x for germline samples. After demultiplexing was performed using bcl2fastq (Illumina, USA), FASTQ files were aligned to the GRCh38 human reference genome using BWA-MEM (v0.7.15). PCR duplicates were marked using Novosort (v1.03.01) and base quality score recalibration was performed using GATK BQSR (v4.1.0). The aligned BAM files were subjected to single nucleotide variant (SNV) analyses using MuTect2 (GATK v4.0.5.1), Strelka2 (v2.9.3), and Lancet (v1.0.7). SNVs were annotated as high confidence if they were reported by at least two variant callers.[1]

WGS analysis of plasma-derived cell-free DNA and contrived DNA
Plasma cfDNA and contrived DNA obtained from fragmented matched tumor and germline cell lines were quantified using the Qubit dsDNA High-Sensitivity Assay (Thermo Fisher, USA). WGS libraries were prepared from plasma cell-free DNA or contrived DNA using a target of 10 ng of DNA through end-repair, A-tailing, and adapter ligation with custom molecular barcoded adapters.[2] Subsequently, these libraries were amplified through 5 cycles of PCR, pooled, and sequenced with 150 bp paired-end reads using the Illumina NovaSeq6000 platform (Illumina, USA) to a target depth of 30x. After demultiplexing was performed, FASTQ files were quality trimmed using Trimmomatic (v0.33) and aligned to the hg19 human reference genome using BWA-MEM2 (v2.2.1). Somatic variant identification was performed using VariantDx (v11.0.0), which has demonstrated high accuracy for somatic mutation detection and differentiating technical artifacts to enable analyses of SNVs.[3,4]

Detection of ctDNA through integrated WGS analysis

Initially, to ensure that the tumor, germline, and plasma WGS datasets were derived from the same subject, an analysis was performed across 10000 common single nucleotide polymorphisms. Then, a quality control analysis was performed using Picard (v2.18.14) and required ≥20x sequencing depth with a median insert size ≥150 bp for cfDNA samples, ≥40x sequencing depth for tumor samples, and ≥20x sequencing depth for germline samples. Tumor-specific SNVs were filtered to a candidate somatic mutation set by removing: (1) variants observed in the 1000 Genomes (Phase 3) or gnomAD (r2.0.1) population databases, (2) variants overlapping the hg19 UCSC simple tandem repeats track, (3) positions with <10x depth in the tumor or matched normal, (4) positions with an alternate allele count <4 in the tumor or >1 in the matched germline, and (5) variants with a tumor VAF <0.05 (more strict filtering was applied to T>C/A>G variants, which were removed if the tumor VAF was <0.20 or the alternate allele count was <10). Additional variant filtering was performed through generation of a blacklist, where variants were further removed if present (1) in >10% of noncancerous donors or (2) any noncancerous donor contained the variant with ≥25% VAF across a cohort of 20 noncancerous donor plasma samples evaluated in quadruplicate. The final candidate tumor-specific variant set was then compared to the matched test sample unfiltered variant results. Candidate tumor-specific SNVs identified in the test sample were scored (ranging from 0 to 1) using a random forest machine learning algorithm trained using the *caret* package (v6.0.90) within the R statistical computing environment (v4.1.1), independently of the PROVENC3 cohort. To avoid overfitting, model training utilized 5-fold cross validation and limited the number of selected variables per split procedure (hyperparameter *mtry*) to the square-root of the total number of input features. Variants present in properly paired mapped fragments with a random forest score >0.25 were further assessed, requiring an alternate read mapping quality ≥30 and a read-based mutation rate ≤5. The individual variant random forest scores were then aggregated and normalized based on the total number of tumor-specific SNVs assessed. The normalized random forest score (NRFS) was then compared to the noncancerous donor cohort, and a cut-off of one standard deviation above the maximum observed NRFS was required to report an individual test sample as having evidence of the tumor-specific variants. An estimated tumor fraction (termed “Aggregate ctDNA VAF”) was then calculated for each positive test sample based on the aggregate variant allele observations observed as a proportion of the total unique coverage of all individual tumor-specific variants assessed.

Labcorp plasma detect ctDNA test analytical performance

The Labcorp Plasma Detect ctDNA assay involves integrated WGS analyses of patient-matched FFPE tumor tissue DNA, WBC-derived germline DNA and plasma cfDNA (Supplementary Figure 2). Analytical sensitivity studies were performed using contrived reference models derived from five commercially available cell lines, including lung cancer (n=1), breast cancer (n=3), and melanoma (n=1); and commercially procured clinical samples, including nine colorectal cancer and five head and neck cancer patients. The contrived samples were generated from three cell lines (COLO-829, HCC-1187, and HCC-1143) and evaluated in triplicate at 10%, 1%, 0.10%, 0.05%, 0.02%, 0.01%, 0.005%, and 0.001% tumor content. An additional contrived sample series (HCC-1187, HCC-1954) was generated and evaluated in triplicate at 0.05%, 0.01%, 0.005%, and 0.001% tumor content, along with the external contrived reference control sample (SeraSeq gDNA TMB-mix Score 26) evaluated at 0.05% (n=7), 0.01% (n=2), 0.005% (n=3), and 0.001% (n=3) tumor content to increase the number of datapoints near the expected limit of detection. Through a regression analysis of these data, a 95% limit of detection of 0.005% tumor content and a 50% limit of detection of 0.001% tumor content was demonstrated (Supplementary Figure 2B; Supplementary Tables 2 and 3). Analytical specificity studies demonstrated a specificity of 99.6% (2,015/2,023) across 119 noncancerous donor plasma specimens evaluated against 17 reference whole-genome somatic mutation datasets (Supplementary Figure 2C; Supplementary Tables 2 and 3). Analysis of an external contrived reference control sample (SeraSeq gDNA TMB-mix Score 26; 0.05% tumor content) demonstrated highly reproducible results for the estimated tumor fraction across 45 independent sequencing runs evaluated for the PROVENC3 clinical study cohort (n=45 runs, coefficient of variation 7.2%; Supplementary Figure 2D). A median of 5108 (IQR 3776-7411) high confidence tumor-specific single nucleotide variants were identified per patient in the PROVENC3 cohort, consistent with previous studies[5,6], which were utilized for plasma ctDNA detection (Supplementary Table 4).

References

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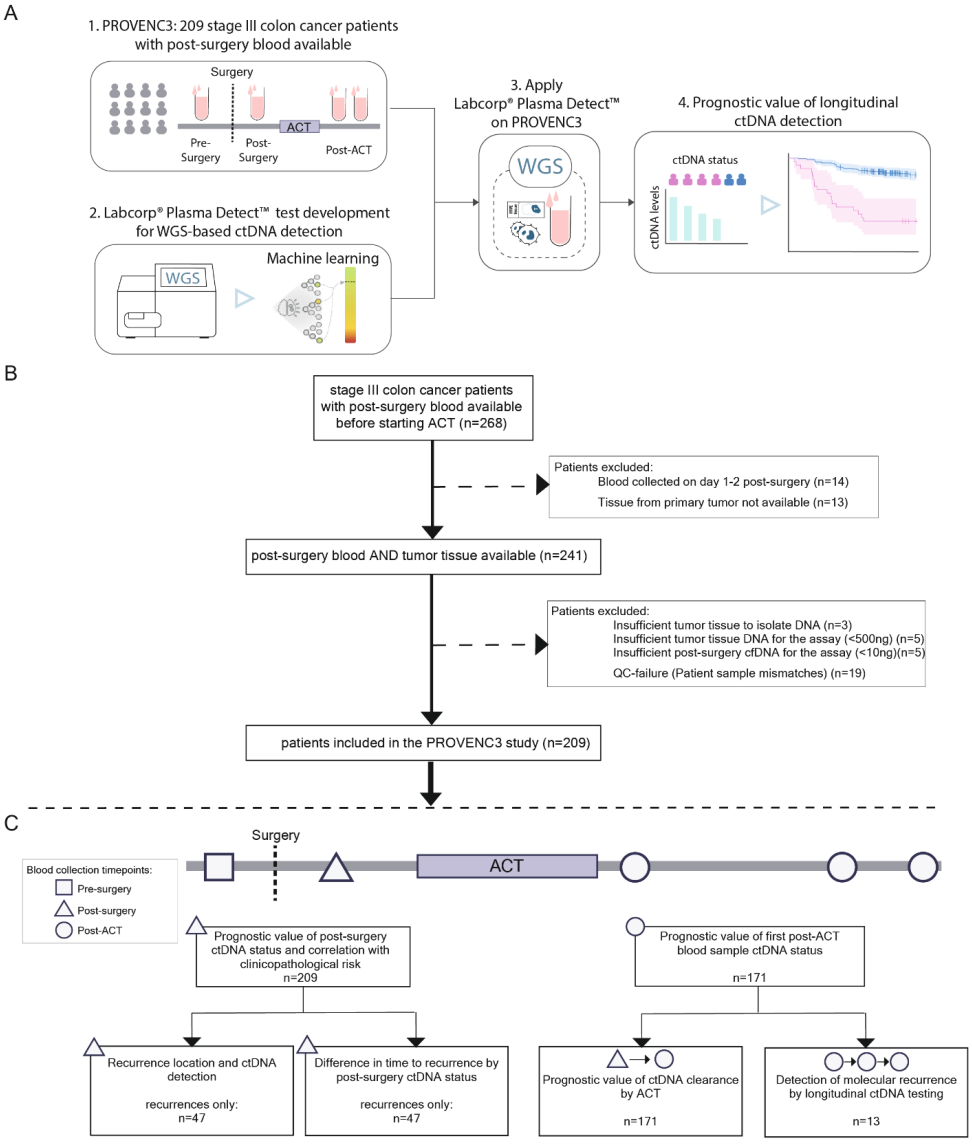
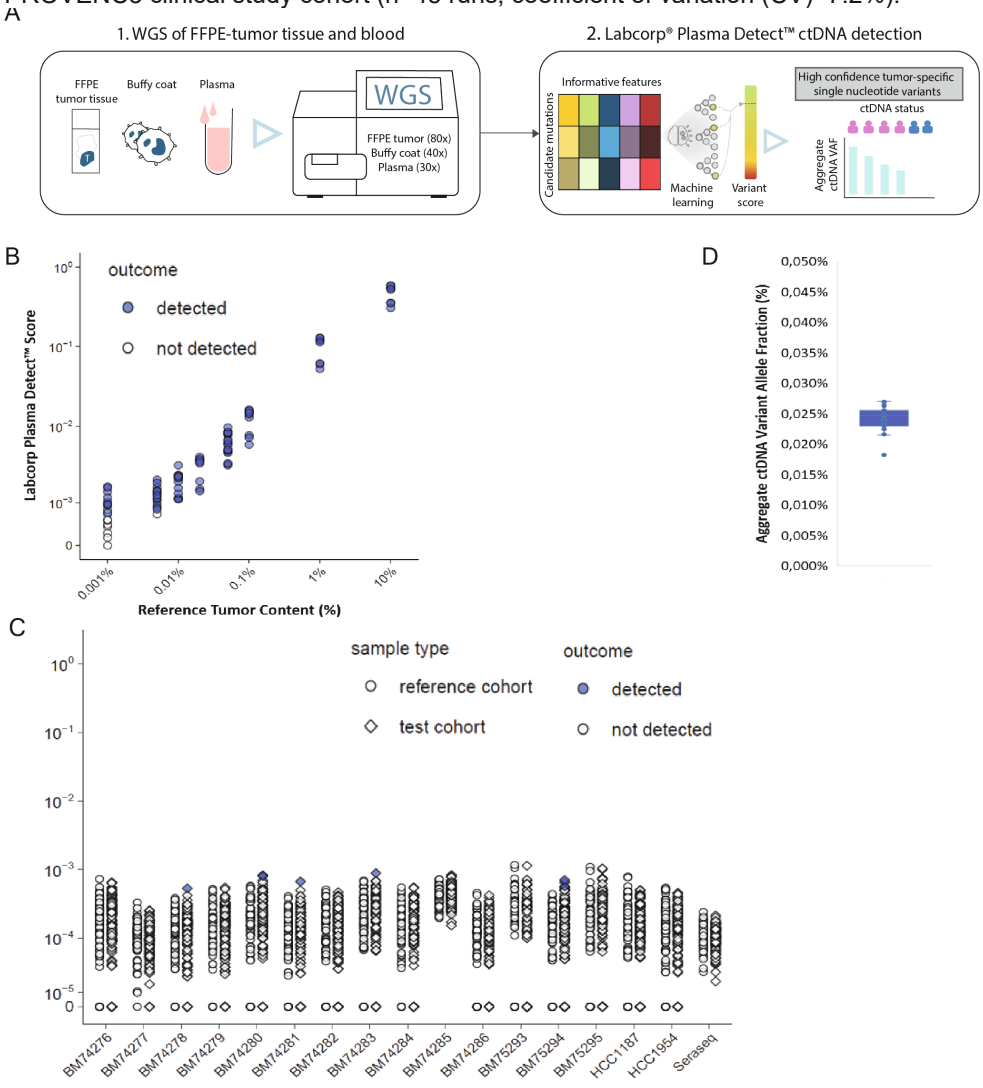
5. Sjoblom T, Jones S, Wood LD, et al. The consensus coding sequences of human breast and colorectal cancers. *Science.* 2006;314(5797):268-274.
6. Wang TL, Diaz LA Jr, Romans K, et al. Prevalence of somatic alterations in the colorectal cancer cell genome. *Proc Natl Acad Sci U S A.* 2002;99(5):3076-3080.

Supplementary figures

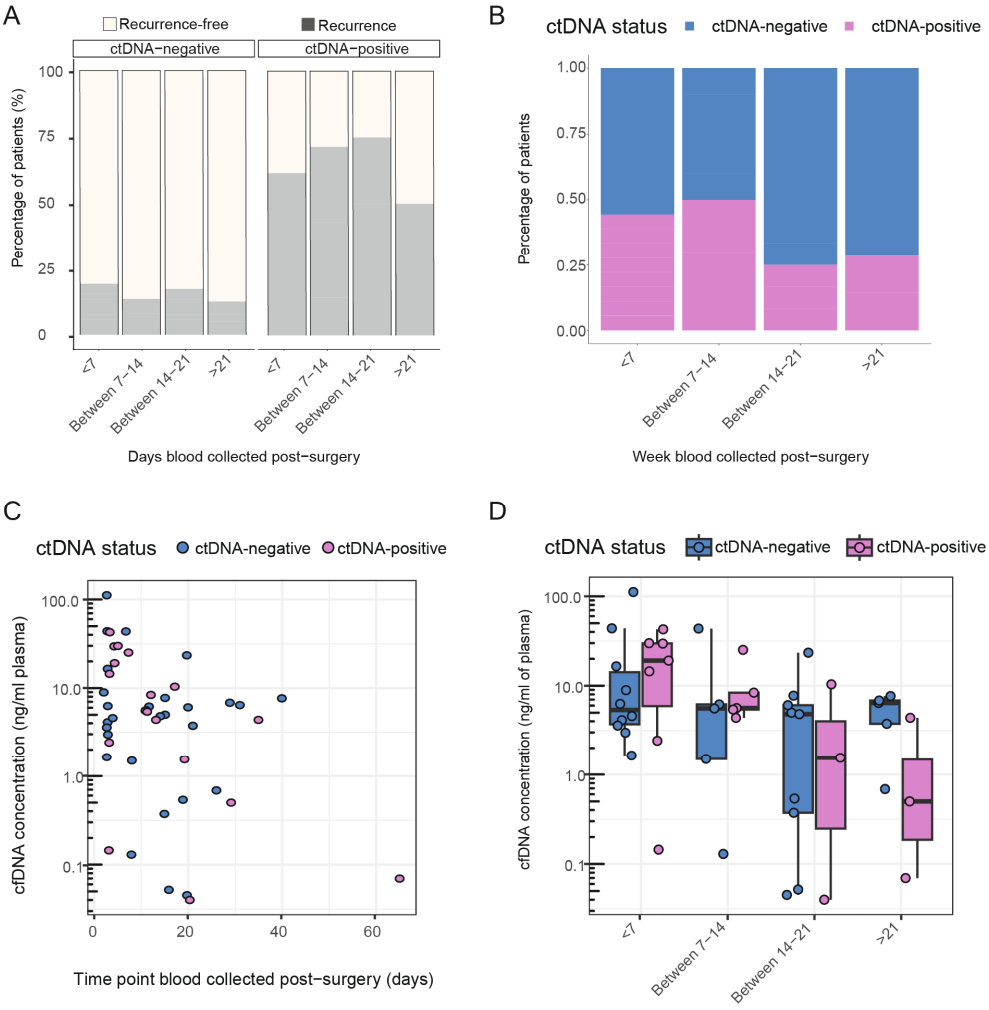
Supplementary Figure 1: PROVENC3 study overview. A) Study overview. B) Selection of the 209 patients of the PROVENC3 clinical study population and the main exclusion criteria from final analysis. C) Overview of the number of patients analyzed for each research question. Each rectangle contains a research question evaluated, and the number of patients and ctDNA time points evaluated.
ACT, adjuvant chemotherapy; QC, quality control; WGS, whole genome sequencing.



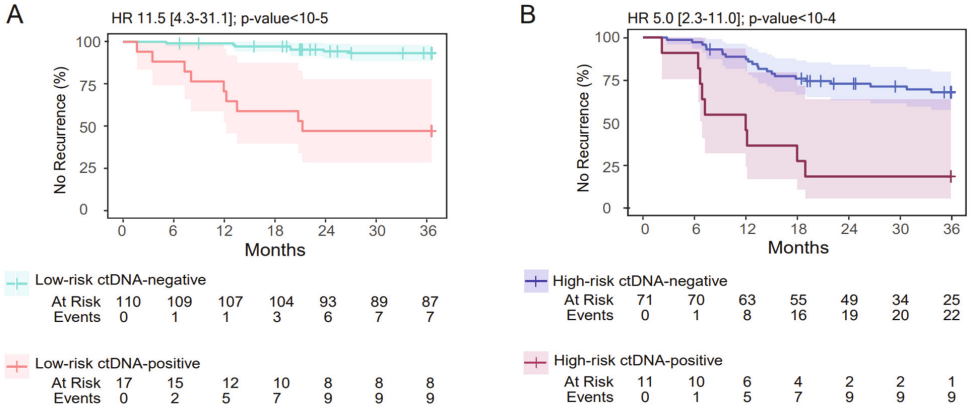
Supplementary Figure 2: A) Schematic overview of the Labcorp Plasma Detect test workflow. B) Analytical sensitivity studies were conducted across five commercially available contrived cell line samples at 10%, 1%, 0.10%, 0.05%, 0.02%, 0.01%, 0.005%, and 0.001% tumor content. C) Analytical specificity was evaluated across 119 noncancerous donor plasma specimens against 17 reference whole genome somatic mutation datasets. D) Reproducibility analysis of an external contrived reference control sample (0.05% tumor content) across 45 independent runs evaluated for the PROVENC3 clinical study cohort (n=45 runs, coefficient of variation (CV)=7.2%).



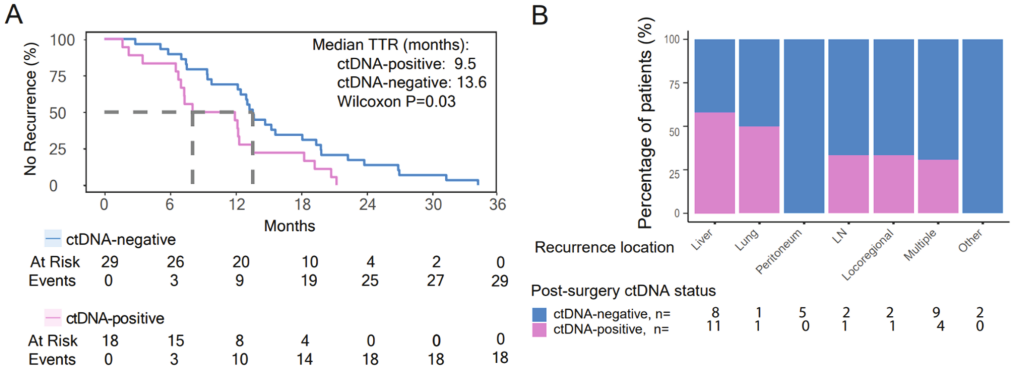
Supplementary Figure 3: Post-surgery ctDNA status and cfDNA concentration in context of timing of the landmark post-surgery blood draw. A) Percentage of patients experiencing a recurrence per time window of post-surgery blood draw, stratified by ctDNA status post-surgery (n=209). B) Percentage of the 47 patients experiencing a recurrence per time window of post-surgery blood draw. C) Overview of cfDNA concentration (ng/mL of plasma) compared across different time windows for 47 patients who experienced a recurrence. D) cfDNA concentration stratified by ctDNA status across different time windows for 47 patients who experienced a recurrence. *cfDNA, cell free DNA; mL, milliliter; ng, nanograms.*



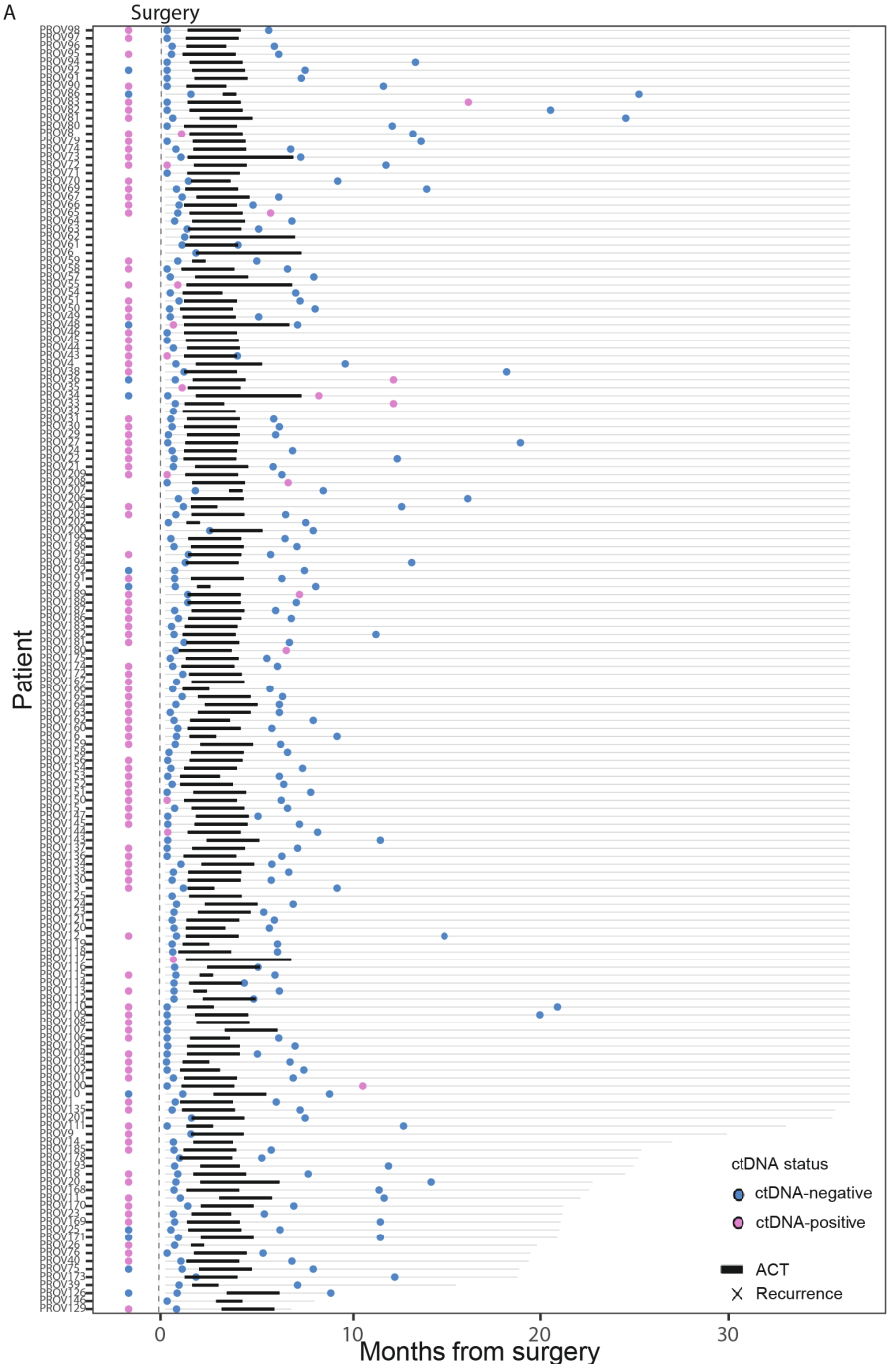
Supplementary Figure 4: TTR for pathological low-risk and high-risk groups, stratified by post-surgery ctDNA status. a) Kaplan-Meier estimate for Cox regression analyses for pathological low-risk stage III colon cancer patients stratified by post-surgery ctDNA status, including confidence intervals. Censored patients are indicated with a vertical line. b) Kaplan-Meier estimate for Cox regression analyses for pathological high-risk stage III colon cancer patients stratified by post-surgery ctDNA status, including confidence intervals. Censored patients are indicated with a vertical line.
CI, confidence interval; ctDNA, circulating tumor DNA; HR, hazard ratio; TTR, time to recurrence.

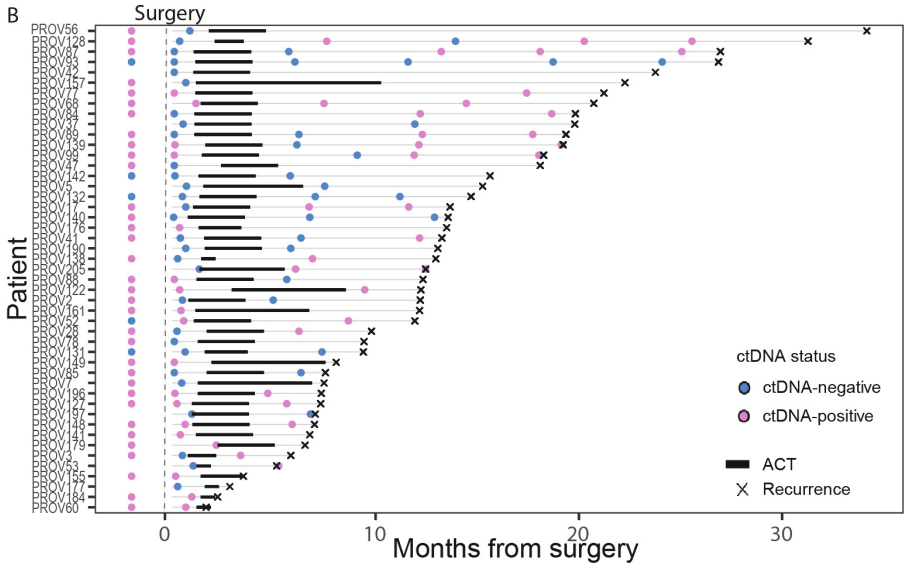


Supplementary Figure 5: Post-surgery ctDNA status in patients experiencing a recurrence. A) Kaplan-Meier estimate for time to recurrence, stratified by post-surgery ctDNA status for all patients experiencing disease recurrence (n=47). B) Post-surgery ctDNA status per recurrence location (n=47).
ACT, adjuvant chemotherapy; ctDNA, circulating tumor DNA; TTR, time to recurrence



Supplementary Figure 6: Swimmer plot of all samples analyzed for patients who remained A) recurrence-free or B) experienced a recurrence during the follow-up reported. *ACT, adjuvant chemotherapy; ctDNA, circulating tumor DNA.*





Supplementary tables

Supplementary Table 2: Summary of whole genome sequencing QC metrics for cell line, tumor, and germline analytical study cohort.
Available online in the manuscript.

Supplementary Table 3: Summary of whole genome sequencing QC metrics for cell-free and contrived DNA analytical study cohort.
Available online in the manuscript.

Supplementary Table 4: Summary of whole genome sequencing QC metrics for the PROVENC3 tumor and germline cohort.
Available online in the manuscript.

Supplementary Table 5. Summary of whole genome sequencing QC metrics for the PROVENC3 plasma cohort.
Available online in the manuscript.

Supplementary Table 1: Summary of baseline clinicopathological characteristics for the PROVENC3 cohort. Baseline characteristics and ACT type received stratified by ctDNA status post-surgery. Fisher exact test, significance level 0.05. *ctDNA, circulating tumor DNA; MSI, microsatellite instability; MSS, microsatellite stability; mut, mutant'; WT, wild type.*

* 89% of the patients receiving CAPOX 1-4 cycles received 4 cycles.

** 60% of the patients receiving CAPOX 5-8 cycles received 8 cycles.

	Total		**ctDNA-positive**		**ctDNA-negative**		
	n	**%**	**n**	**%**	**n**	**%**	**p-value**
	209	**100**	**28**	**100**	**181**		
Age median [min-max]	63 [32-83]		68 [43-83]		63 [32-79]		0.11
Sex							0.15
Female	97	46	9	32	88	49	
Male	112	54	19	68	93	51	
Pathological risk							1
Low risk	127	61	17	61	110	61	
High risk	82	39	11	39	71	39	
T status							1
T4	51	24	7	25	44	24	
T1-3	158	76	21	75	137	76	
T1	4	2	0	0	4	2	
T2	29	14	3	11	26	14	
T3	125	60	18	64	107	59	
N status							0.49
N1	154	74	19	68	135	75	
N1a	54	26	6	21	48	27	
N1b	80	38	11	39	69	38	
N1c	11	5	2	7	9	5	
N1m	9	4	0	0	9	5	
N2	55	26	9	32	46	25	
N2a	31	15	5	18	26	14	
N2b	24	11	4	14	20	11	
MSI status							0.38
Stable (MSS)	179	86	26	93	153	85	
Instable (MSI)	30	14	2	7	28	15	
Resection							0.043
Radical	201	96.2	24	95.7	177	97.8	
Non-radical	3	1.4	2	7.1	1	0.6	
Missing	5	2.4	2	7.1	3	1.7	
Histology							0.63
Adenocarcinoma	185	89	26	93	159	88	
Mucinous AC	18	9	1	4	17	9	
Medullary, NOS	3	1	0	0	3	2	
Signet ring cell	3	1	1	4	2	1	
Differentiation grade							1
Well differentiated	0	0	0	0	0	0	
Moderately	171	82	22	79	149	82	

Poorly	29	14	4	14	25	14	
Undifferentiated	0	0	0	0	0	0	
Missing	9	4	2	7	7	4	
Tumor location							0.31
Left	119	57	13	46	106	59	
Right	90	43	15	54	75	41	
Angioinvasion							0.66
Extramural	40	19	6	21	34	19	
Intramural	6	3	1	4	5	3	
No	108	52	13	46	95	52	
Missing	55	26	8	29	47	26	
RAS				0			0.15
WT	126	60	13	46	113	62	
mut	83	40	15	54	68	38	
BRAF							0.46
WT	167	80	21	75	146	81	
mut	42	20	7	25	35	19	
ACT regimen							
CAPOX 1-4 cycles*	188	90	20	71	168	93	
CAPOX 5-8 cycles**	9	4	2	7	7	4	
FOLFOX 8 cycles	1	1	0	0	1	1	
Capecitabine 1-7	4	2	2	7	2	1	
Capecitabine 8 cycles	7	3	4	14	3	2	

Supplementary Table 6: Univariable Cox analyses for post-surgery ctDNA, pathological risk factors and MSI status.
CI, confidence interval; ctDNA, circulating tumor DNA; HR, hazard ratio; MSI, microsatellite instability; MSS, microsatellite stability; N, node; T, tumor.

Risk variable	Level	n	HR	95%CI	p-value
Post-surgery ctDNA status	ctDNA-negative	181	ref		
	ctDNA-positive	28	6.17	[3.42-11.16]	1.64e-09
Pathological risk	Low risk	127	ref		
	High risk	82	3.54	[1.94-6.49]	4.12e-05
T status	T1-3	158	ref		
	T4	51	2.99	[1.68-5.32]	0.000193
N status	N1	154	ref		
	N2	55	3.17	[1.79-5.62]	8.04e-05
MSI status	Stable (MSS)	179	ref		
	Unstable (MSI)	30	0.37	[0.11-1.19]	0.09

Supplementary Table 7: Univariable Cox regression analyses per individual pathological risk variable and MSI status, stratified for ctDNA status. "ctDNA status" corresponds to the post-surgery time point.
CI, confidence interval; ctDNA, circulating tumor DNA; MSI, microsatellite instability; MSS, microsatellite stability; N, Node; Ref, Reference level; T, Tumor.

Risk variable	Level	ctDNA status	HR	95%CI	p-value
Pathological risk + Post-surgery ctDNA status	Low risk	ctDNA-negative	Ref		
	Low risk	ctDNA-positive	11.74	[4.36-31.56]	1.06e-06
	High risk	ctDNA-negative	5.65	[2.41-13.22]	6.70e-05
	High risk	ctDNA-positive	28.45	[10.48-77.17]	4.84e-11
T status + Post-surgery ctDNA status	T1-3	ctDNA-negative	Ref		
	T1-3	ctDNA-positive	6.43	[2.95-14.03]	2.85e-06
	T4	ctDNA-negative	3.28	[1.58-6.81]	0.00139
	T4	ctDNA-positive	39.10	[15.11-101.1]	4.16e-14
N status + Post-surgery ctDNA status	N1	ctDNA-negative	Ref		
	N1	ctDNA-positive	8.70	[3.89-19.45]	1.36e-07
	N2	ctDNA-negative	4.14	[1.99-8.16]	0.00014 3
	N2	ctDNA-positive	16.79	[6.63-42.53]	2.69e-09
MSI status + Post-surgery ctDNA status	MSI	ctDNA-negative	Ref		
	MSI	ctDNA-positive	7.11	[0.64-78.43]	0.11
	MSS	ctDNA-negative	2.59	[0.61-10.93]	0.19
	MSS	ctDNA-positive	15.42	[3.55-66.94]	0.00026

Supplementary Table 8: Likelihood Ratio Test for model goodness-of-fit assessment. Multivariable Cox models were fitted including different pathological variables and MSI status. Four likelihood ratio tests were performed to assess the added value of ctDNA status in each model. ctDNA status corresponds to the post-surgery time point.
ctDNA, circulating tumor DNA; LRT, likelihood ratio test; MSI, microsatellite instability; MSS, microsatellite stability; N, node; T, tumor.

	Predictor combinations in Cox model	p-value	Better model
LRT1	Pathological_Risk		
	Pathological_Risk + **ctDNA status**	1.71e-08	Pathological_Risk + **ctDNA status**
LRT2	Pathological_Risk + MSI status		
	Pathological_Risk + MSI + **ctDNA status**	3.91e-08	Pathological_Risk + MSI + **ctDNA**
LRT3	T status + N status		
	T status + N status + **ctDNA status**	6.34e-09	T status + N status + **ctDNA**
LRT4	T status + N status + MSI status		
	T status + N status + MSI status + **ctDNA**	1.06e-08	T status + N status + MSI + **ctDNA**

Supplementary Table 9: Multivariable Cox model analysis with hazard ratio per variable included. ctDNA status corresponds to the post-surgery time point.
CI, confidence interval; ctDNA, circulating tumor DNA; HR, hazard ratio; MSI, microsatellite instability; MSS, microsatellite stability; N, Node; T, Tumor.

Model	Covariate	Reference	HR	95% CI	p-value
Pathological_Risk+	High-risk	Low-risk	3.79	[2.06-6.96]	1.78e-05
MSI status+	MSS	MSI	0.73	[0.63-6.74]	0.23
ctDNA status	ctDNA-positive	ctDNA-negative	6.55	[3.60-11.92]	7.61e-10
T status +	T4	T1-3	3.22	[1.72-6.05]	0.000264
N status +	N2	N1	2.73	[1.17-4.00]	0.0127
MSI status +	MSS	MSI	1.99	[0.61-6.56]	0.253
ctDNA status	ctDNA-positive	ctDNA-negative	7.53	[4.03-14.05]	2.35e-10

5

Added value of tumor-stroma ratio to post-surgery circulating tumor DNA and pTN stagein risk stratification of patients with stage III colon cancer treated with adjuvant chemotherapy

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Under review

Abstract

Introduction

Patients with stage III colon cancer (CC) are routinely treated with resection followed by adjuvant chemotherapy (ACT). Half of patients are cured by surgery alone and overtreated with ACT, yet another ~30% experience disease recurrence despite ACT. Upfront risk stratification requires biomarkers beyond the conventional pathological stage (pTN). Detection of post-surgery circulating tumor DNA (ctDNA) is indicative of minimal residual disease and prognostic of disease recurrence, but its negative predictive value is insufficient to guide treatment de-escalation. This study aimed to investigate whether adding tumor-stroma ratio (TSR) can improve stratification.

Methods

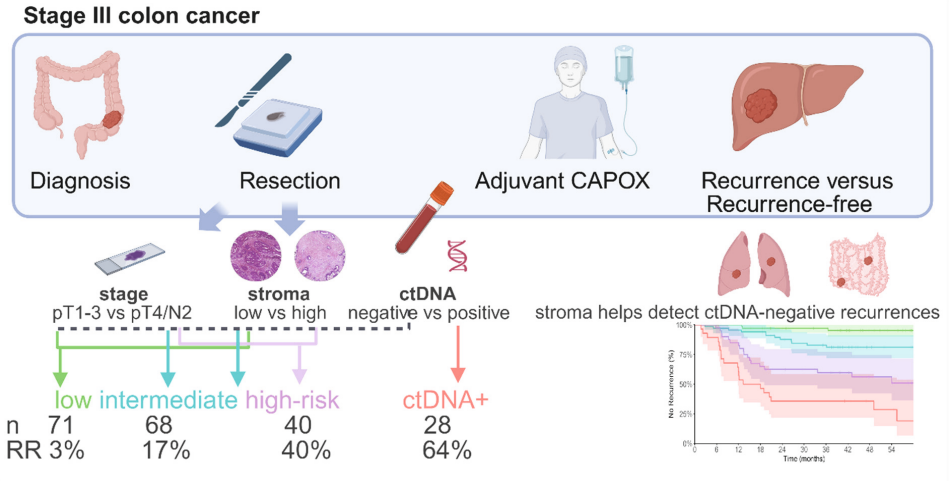
This study included 207 patients from the PLCRC-PROVENC3 with radical resection of stage III CC followed by ACT. Post-surgery ctDNA status was determined using Labcorp® Plasma Detect™. On a H&E resection section, the TSR was scored by trained observers and dichotomized as stroma-low (≤50% stroma) versus stroma-high (>50%). The primary outcome was time to recurrence in univariable and multivariable Cox analyses, reporting 3-year recurrence risk (RR) to inform risk-group stratification.

Results

Post-surgery ctDNA was the strongest predictor (n=28, 3-year RR 64.3% [95%CI 41.3-78.3] versus 16.9% [11.1-22.4], HR 5.8 [3.3-10.1]), followed by pT4 and/or pN2 (n=82, HR 2.9 [1.7-5.0]) and a stroma-high tumor (n=88, HR 2.7 [1.5-4.6]). Within the ctDNA-negative subgroup (n=179), we identified a low-risk group (pT1-3N1 and stroma-low; n=71, 3-year RR 2.9% [0-6.8]), intermediate-risk group (either pT4/N2 or stroma-high; n=68, 3-year RR 17.2% [7.4-26.0], HR 5.4 [1.6-18.9]), and high-risk group (pT4/N2 and stroma-high; n=40, 3-year RR 40.3% [22.9-53.9], HR 14 [4.2-47.8]).

Conclusions

Adding TSR to post-surgery ctDNA and pTN stage improved risk stratification of stage III CC patients receiving ACT. One-third of the patients had none of the biomarkers and could be considered for de-escalation based on their very low RR. In addition to ctDNA-positive patients, ctDNA-negative patients with a pT4/N2 stroma-high tumor may require treatment escalation to reduce their high RR.


Stage III colon cancer
Diagnosis
Resection
Adjuvant CAPOX
Recurrence versus Recurrence-free
stage
pT1-3 vs pT4/N2
stroma
low vs high
ctDNA
negative vs positive
stroma helps detect ctDNA-negative recurrences
low intermediate high-risk
ctDNA+
n 71 68 40 28
RR 3% 17% 40% 64%
No Recurrence (%)
Time (months)

Introduction

Colon cancer is the third most prevalent cancer globally and the second leading cause of cancer-related mortality. Patients with stage III colon cancer (CC) are recommended surgical resection followed by adjuvant chemotherapy (ACT).[1] Worldwide, the standard ACT consists of a fluoropyrimidine combined with oxaliplatin for patients deemed fit for treatment. In the Netherlands, the preferred regimen is capecitabine with oxaliplatin (CAPOX).[2] Of all stage III CC patients, 50% would have been cured by surgery only and these patients are currently overtreated with ACT.[3] On the other hand, 30% of the patients experience recurrence of disease despite ACT and are in need of improved adjuvant treatment. A better identification of these respective low- and high-risk groups is warranted to enable individualized ACT recommendations. This highlights the need for biomarkers to improve risk stratification, beyond the current definition of low-risk (pT1-3N1) and high-risk (pT4 and/or N2) stage III CC based on TNM stage.[4]

Circulating tumor DNA (ctDNA) allows for the detection and monitoring of minimal residual disease (MRD) after resection of the tumor. Several studies demonstrated that ctDNA detection post-surgery has a strong prognostic value for an increased risk of recurrence (RR) in stage III CC patients treated with ACT.[5-9] However, in order to use negative ctDNA results to guide clinical decisions towards de-escalation of treatment, false negative ctDNA results remain a concern. To improve negative predictive value, combination of ctDNA with other biomarkers is needed.[10,11] Especially characteristics of the resected tumor tissue, involving both the tumor compartment[12] and/or the tumor microenvironment (TME)[13], hold potential to serve as additional prognostic biomarkers. A high level of stroma is associated with tumor progression, metastasis, and resistance to therapy.[14-16] The tumor-stroma-ratio (TSR) quantifies the percentage of stroma in the resected tumor, based on a diagnostic histopathology slide.[17,18] Recent findings from the UNITED study validated the TSR as an independent prognostic factor in stage II/III CC, also in the group treated with ACT.[19]

The objective of this study is to investigate the added value of TSR to post-surgery ctDNA and pTN stage in risk stratification of stage III colon cancer. We hypothesize that the combination of pT4/N2 and stroma-high enables the identification of patients at high risk of recurrence despite no detected ctDNA post-surgery, while absence of all these risk factors helps define a very-low risk group.

Methods

Study design and participants

Patients provided written informed consent in the Prospective Dutch Colorectal Cancer cohort (PLCRC; METC 12-510, NCT02070146). PLCRC is a nationwide study that provides the infrastructure for collection of clinical data, blood and tissue.[20] For the observational PLCRC-substudy PROVENC3[7], we selected 207 patients who were diagnosed with stage III CC between 2016 and 2021 and treated with ACT (n=196 CAPOX, n=11 capecitabine). Exclusion criteria were incomplete resection (R1 or R2), neoadjuvant treatment or prior malignancy in the past five years. Clinical data was collected by trained data managers in the Netherlands Cancer Registry (NCR). Pathological tumor and node staging was based on the 8th edition of the TNM classification by the American Joint Committee on Cancer.

ctDNA detection

In PLCRC-PROVENC3, a post-surgery blood sample was collected at least four days after surgery and before initiation of ACT.[7] ctDNA status was determined using Labcorp® Plasma Detect™, a tumor tissue-informed approach involving integrated whole genome sequencing analyses of plasma cell-free DNA (30x), germline DNA (40x) and tumor tissue DNA (80x). To this end, formalin-fixed paraffin-embedded (FFPE) tumor blocks were obtained through the Dutch Nationwide Pathology Databank (Palga).[21] Patients and treating physicians remained blinded for the ctDNA result.

TSR scoring

The tumor tissue blocks used for DNA isolation were also used to generate a 4 µM slide, which was stained with hematoxylin and eosin (H&E) and digitized (NanoZoomerXR Hamamatsu, 40x). Two trained observers (F.H. and W.M.), who were unaware of the patient outcomes and ctDNA results, scored the TSR according to the previously described method.[18] At low magnification, the most invasive tumor area with the highest representation of stroma was selected on each slide. Subsequently, at 10x objective (100x magnification) stromal percentage was scored at 10% increments. Necrosis, mucin, vascular structures, glandular lumen, or muscle tissue were excluded from the scored area. Tumors were categorized as stroma-low (≤50% stroma) or stroma-high (>50% stroma) (examples in Supplementary Figure 1A), using the predefined cut-off value with most discriminative power.[15] In cases with no agreement

between the independent observers, consensus was reached through discussion. For most cases, one diagnostic slide was available. In instances with multiple slides, the slide with the highest TSR was used as previously validated.[16]

Statistical analysis

Proportions of discordant classifications between ctDNA and TSR were estimated, along with their 95% confidence intervals (95% CIs), using the Wilson method. Biomarker-based subgroups were compared based on categorical and continuous variables, using the χ^2 test and independent samples t-test, respectively. Median follow-up from resection was reported with interquartile range (IQR). The primary endpoint was time to recurrence (TTR), defined as the first incidence of locoregional or distant recurrence and censored at last date of standard clinical follow-up. The secondary endpoint was overall survival (OS), derived from the national municipal population registry in January 2025. TTR and OS were assessed in univariable log-rank and multivariable Cox proportional hazards models, after checking that assumptions were not violated visibly or based on Schoenfeld residuals. Per biomarker or risk group, the hazard ratio (HR) and 3-year recurrence rate and 5-year OS rate were reported with 95%CIs. Statistical analyses were performed using R studio (v4.2.2). Two-sided p-values <0.05 were considered statistically significant.

Results

Description of the cohort and the biomarkers

A total of 207 patients with radical resection and adjuvant CAPOX (95%) or capecitabine (5%) were included in the analyses. Post-surgery blood samples were available at a median of 13 days after surgery [IQR 4-20] and ctDNA was detected in 28 patients (13.5%). The TSR was scored with a Cohen's Kappa of 0.86, indicating a strong agreement between both observers. The TSR was classified as stroma-high in 88 out of 207 patients (42.5%). When stratified on ctDNA status, 39.3% [95%CI 23.6-56.7] of ctDNA-positive patients and 43.0% [36.0-50.3] of ctDNA-negative patients were stroma-high (p=0.710). This results in a low overall concordance between ctDNA and TSR of 54.6% [47.8-61.2] (Supplementary Figure 1B). Pathological T4 and/or N2 stage was observed in 39.6% of patients and was correlated with a stroma-high tumor (p=0.002) but not with ctDNA detection (p=0.687). Other patient and tumor characteristics did not differ significantly across ctDNA and TSR status (Table 1).

Table 1: Patient and tumor characteristics in the overall cohort and per biomarker.
CI, confidence interval; ctDNA, circulating tumor DNA; MSS/I, microsatellite stable/instable; n, number of patients; pTN, pathological T and N stage.

		ctDNA-negative		ctDNA-positive	
	Overall (n=207)	**stroma-low (n=102)**	**stroma-high (n=77)**	**stroma-low (n=17)**	**stroma-high (n=11)**
Age Mean (SD)	63.1 (9.4)	62.8 (9.1)	62.6 (9.7)	63.6 (10.4)	69.3 (6.2)
Sex					
Female	116 (56.0%)	54 (52.9%)	32 (41.6%)	6 (35.3%)	3 (27.3%)
Male	91 (44.0%)	48 (47.1%)	45 (58.4%)	11 (64.7%)	8 (72.7%)
pTN stage					
T1-3N1	125 (60.4%)	71 (69.6%)	37 (48.1%)	12 (70.6%)	5 (45.5%)
T4/N2	82 (39.6%)	31 (30.4%)	40 (51.9%)	5 (29.4%)	6 (54.5%)
Tumor sidedness					
Left	125 (60.4%)	58 (56.9%)	46 (59.7%)	7 (41.2%)	5 (45.5%)
Right	82 (39.6%)	44 (43.1%)	31 (40.3%)	10 (58.8%)	6 (54.5%)
Microsatellite Stability					
Stable (MSS)	175 (84.5%)	83 (81.4%)	66 (85.7%)	15 (88.2%)	11 (100%)
Instable (MSI)	32 (15.5%)	19 (18.6%)	11 (14.3%)	2 (11.8%)	0 (0%)
Lymphatic and/or angioinvasion					
No	91 (46.9%)	46 (51.1%)	33 (43.4%)	8 (47.1%)	4 (36.4%)
Yes	103 (53.1%)	44 (48.9%)	43 (56.6%)	9 (52.9%)	7 (63.6%)
Missing	13	12	1	0	0
Histology					
Adenocarcinoma	182 (88.3%)	91 (89.2%)	65 (85.8%)	15 (88.2%)	11 (100%)
Other	24 (11.7%)	11 (10.8%)	11 (14.2%)	2 (11.8%)	0 (0%)
Missing	1	0	1	0	0
Differentiation					
Moderate	174 (85.3%)	87 (86.1%)	63 (84.0%)	15 (88.2%)	9 (81.8%)
Poor	30 (14.7%)	14 (13.9%)	12 (16.0%)	2 (11.8%)	2 (18.2%)
Missing	3	1	2	0	0
Site of recurrence	**n=56**	**n=9**	**n=27**	**n=11**	**n=9**
Locoregional	5 (8.9%)	1 (11.1%)	3 (11.1%)	1 (9.1%)	0 (0%)
Liver only	21 (37.5%)	3 (33.3%)	6 (22.2%)	6 (54.5%)	6 (66.7%)
Lung only	7 (12.5%)	1 (11.1%)	5 (18.5%)	1 (9.1%)	0 (0%)
Lymph only	2 (3.6%)	0 (0%)	1 (3.7%)	1 (9.1%)	0 (0%)
Peritoneum only	10 (17.9%)	1 (11.1%)	6 (22.2%)	1 (9.1%)	2 (22.2%)
Multiple sites	7 (12.6%)	1 (11.1%)	4 (14.8%)	1 (9.1%)	1 (11.1%)
Other	4 (7.1%)	2 (22.2%)	2 (7.4%)	0 (0%)	0 (0%)

Independent prognostic value of post-surgery ctDNA, pTN stage and TSR

Of all 207 patients, 56 (27.1%) patients experienced a recurrence (median TTR 13.4 months [IQR 7.8-22.3]) and 151 remained recurrence-free (median follow-up 43.9 months [IQR 37.6-59.6]). We first explored univariable prognostic value of patient and tumor characteristics for recurrence of disease (Table 2). Post-surgery ctDNA-positivity was the strongest prognostic marker, identifying a small group of patients at very high risk of recurrence (n=28 (13.5%), 3-year RR 64.3% [41.3-78.3]) compared with ctDNA-negative patients (n=179, 3-year RR 16.9% [11.1-22.4]; HR 5.8 [3.3-10], p<0.001) (Supplementary Figure 1C). Patients with a pT4 and/or N2 tumor (n=82 (39.6%), 3-year RR 38.9% [17.1-48.8]) were at higher risk than patients with a pT1-3N1 tumor (n=125, 3-year RR 13.2% [7.0-19.1]; HR 2.9 [1.7-5.0], p<0.001) (Supplementary Figure 1D). As for the TSR, patients with a stroma-high tumor had an increased risk (n=88 (42.5%), 3-year RR 33.1% [22.5-42.3]) compared to patients with a stroma-low tumor (n=119, 16.0% [8.9-22.5]; HR 2.7 [1.5-4.7], p<0.001) (Supplementary Figure 1E).

Complementary prognostic value of post-surgery ctDNA, pTN stage and TSR

Notably, within the ctDNA-negative group (n=179), 36 (20%) patients experienced recurrence, of whom 27 (75%) were stroma-high (Table 1). Especially recurrence of disease in peritoneum and lung was missed by post-surgery ctDNA, as published before (false negative 60%[22]-80%[23]). Of the ten patients who developed peritoneal metastases, seven were ctDNA-negative after surgery, of whom six had a stroma-high tumor. Of seven patients with lung metastases, six were ctDNA-negative of whom five had a stroma-high tumors. When combined in a multivariable Cox model with other patient and tumor characteristics, the three biomarkers ctDNA (HR 6.3 [3.4-11.3], p<0.001), pTN stage (HR 3.0 [1.7-5.2], p<0.001) and TSR (HR 2.6 [1.5-4.6], p<0.001) maintained prognostic value (Table 2; Supplementary Figure 2).

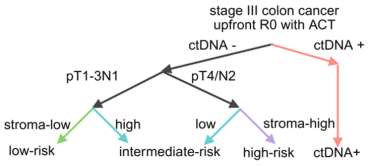
Table 2: post-surgery ctDNA, pathological stage and TSR in univariable and multivariable models for recurrence, adjusting for age, sex, tumor sidedness and microsatellite status. *ctDNA, circulating tumor DNA; HR, hazard ratio; pTN, pathological stage; TSR, tumor-stroma ratio.*

Variable			Univariable			Multivariable		
	Level	**n**	**HR**	**95%CI**	**p-value**	**HR**	**95%CI**	**p-value**
ctDNA	Detected	28	5.8	[3.3-10.1]	<0.001	6.3	[3.4-11.3]	<0.001
pTN stage	pT4/N2	82	2.9	[1.7-5.0]	<0.001	3.0	[1.7-5.2]	<0.001
TSR	Stroma-high	88	2.7	[1.5-4.6]	<0.001	2.6	[1.5-4.6]	<0.001

Combining ctDNA, pTN stage and TSR to stratify patients into relevant risk groups

To stratify patients into clinically relevant subgroups with distinct risk, we combined post-surgery ctDNA, pTN stage and TSR (Figure 1A-B). A low-risk group was defined as ctDNA-negative with none of the tissue-based risk factors: pT1-3N1 and stroma-low. This group consists of 34.3% of all patients (n=71) and these patients had a very low risk of recurrence (3-year RR 2.9% [0-6.8]) after ACT (Figure 1C). ctDNA-negative patients with only one of the other risk factors, either pT4/N2 or stroma-high, showed an intermediate-risk (n=68 (32.9%), 3-year RR 17.2% [7.4-26.0]; HR 5.4 [1.6-18.9], p=0.008). ctDNA-negative patients were tissue-based high-risk in case of both pT4/N2 and stroma-high (n=40 (19.3%), 3-year RR 40.3% [22.9-53.9]; HR 14.1 [4.2-47.8], p<0.001). Highest risk was observed in the patients with ctDNA detection post-surgery, who were therefore considered as separate risk group (n=28 (13.5%), 3-year RR 64.3% [41.3-78.3]; HR 30.4 [9.0-103], p<0.001). Similar to RR, overall survival was better in the low-risk group (5-year OS 95.8% [91.2-100]) compared with the intermediate-risk (5-year OS 84.2% [74.9-94.6], HR 2.7 [0.8-8.5], p=0.099), the tissue-based high-risk group (5-year OS 76.2% [63.5-91.4]; HR 5.8 [1.9-18.0]) and the ctDNA-positive group (5-year OS 64.3% [48.8-85.7]; HR 9.6 [3.2-30.8]) (Figure 1D).

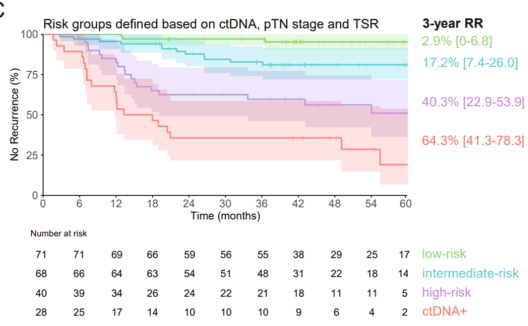
A



B

Risk group	Definition	n (%)	3-year RR	5-year OS
low-risk	ctDNA-, pT1-3N1 and stroma-low	71 (34.3%)	2.9%	95.8%
intermediate-risk	ctDNA-, pT4/N2 or stroma-high	68 (32.9%)	17.2%	84.2%
Tissue-based high-risk	ctDNA-, pT4/N2 and stroma-high	40 (19.3%)	40.3%	76.2%
ctDNA-based high-risk	ctDNA+	28 (13.5%)	64.3%	64.3%

C



D

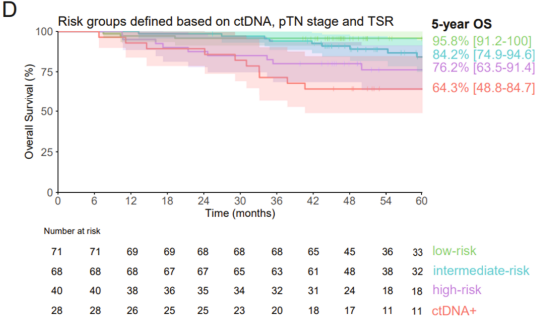


Figure 1: A) Stratification system based on ctDNA, pTN stage and TSR. B) 3-year recurrence risk (RR) per risk group. C) 5-year overall survival (OS) per risk group.

Discussion

This study aimed to evaluate the added value of TSR to post-surgery ctDNA and pTN in stage III CC patients treated with ACT. After confirming univariable and multivariable prognostic significance of all three biomarkers, we used them to stratify patients into clinically relevant risk groups. One-third of patients were considered low risk based on ctDNA-negativity, pT1-3N1 stage and a stroma-low tumor. ctDNA-negative patients with pT4/N2 or stroma-high were intermediate risk, and those with a pT4/N2 and stroma-high tumor were tissue-based high-risk, complementing the ctDNA-based high-risk group.

Our findings support that liquid biopsy ctDNA testing should be combined with other (tissue-based) biomarkers in multimodal assessment of recurrence risk.[11,24] Post-surgery detection of ctDNA indicates the presence of MRD and is the strongest prognostic biomarker to date in stage III CC patients treated with ACT. However, the observed recurrence risk (~20% despite ACT)[5-9] in patients with no detected ctDNA is too high to withhold ACT. Current ctDNA assays fail to detect part of (micro)metastases, especially from low-shedding sites like the lung and peritoneum.[22,23,25,26] Pathological stage reflects the locoregional tumor extent and adding pathological stage helps to delineate a lower-risk ctDNA-negative subgroup based on pT1-3N1.[7,27,28] We now show a further clinically relevant reduction in 3-year RR to less than 3% in the subset of patients with ctDNA-negative, pT1-3N1 stroma-low tumor.

The complementary value of the TSR is evident from the observation that recurrences in ctDNA-negative patients, particularly in the peritoneum and lungs, occurred predominantly in patients with stroma-high primary tumors. The TSR as prognostic biomarker was previously described in the UNITED study, which observed a comparable proportion of stroma-high tumors (39%) and prognostic impact (event rate 34% versus 20%, HR 1.7).[19] These findings, together with the almost perfect agreement between observers, underline the robustness of the TSR. Moreover, the TSR can be easily integrated in the diagnostic workflow, as it can be determined during standard pathological staging on the same H&E slide of the deepest invasive front.[18,29] Therefore, concurrent use of pathological stage and TSR may stratify tissue-based risk within a few days after surgery.

A limitation in the interpretation of the study results is that all patients were treated with ACT, conform the guideline. Although we identify a large subgroup with very low risk after ACT, a control group of patients without ACT is required to inform whether de-escalation of treatment would be safe. Future studies should prospectively validate the proposed risk stratification based on ctDNA, pTN stage and TSR, preferably randomizing the low-risk patients between ACT and surgery only. This may help inform whether addition of ACT (OS >95% in our study) provides a clinically relevant benefit, defined as >5% improvement in OS.[30,31] If the benefit from ACT does not outweigh the potential toxicity, especially neuropathy[32], low-risk patients may decide to withhold ACT until ctDNA becomes detectable in serial monitoring during intensive follow-up.[33] For the high-risk patients, it remains to be investigated what escalation treatment would provide benefit. ctDNA studies show that half to two-thirds of patients with MRD are not cured by adjuvant CAPOX[5-9], and recent results suggest that this is not improved by longer CAPOX duration or escalation to FOLFOXIRI.[34] The shown relevance of stroma, consistent with prior findings on consensus molecular subtype 4[6] and cancer-associated fibroblasts[35], suggest that the TME may provide a potential target for novel treatment options[36].

In conclusion, the combination of ctDNA, pTN stage and TSR holds potential to improve risk stratification of patients with stage III colon cancer, specifically to identify the false negative ctDNA results after surgery. The ctDNA-negative patients with a pT1-3N1 stroma-low tumor were at very low risk of recurrence. These patients may currently be overtreated with ACT and may be considered for de-escalation in the future. Conversely, patients with a stroma-high pT4/N2 tumor, in addition to all ctDNA-positive patients, were at high risk of recurrence despite standard ACT. They may currently be undertreated and may be selected for treatment escalation. Multimodal strategies combining ctDNA and tissue-based biomarkers may help the prediction of risk of recurrence and inform personalized treatment decisions making.

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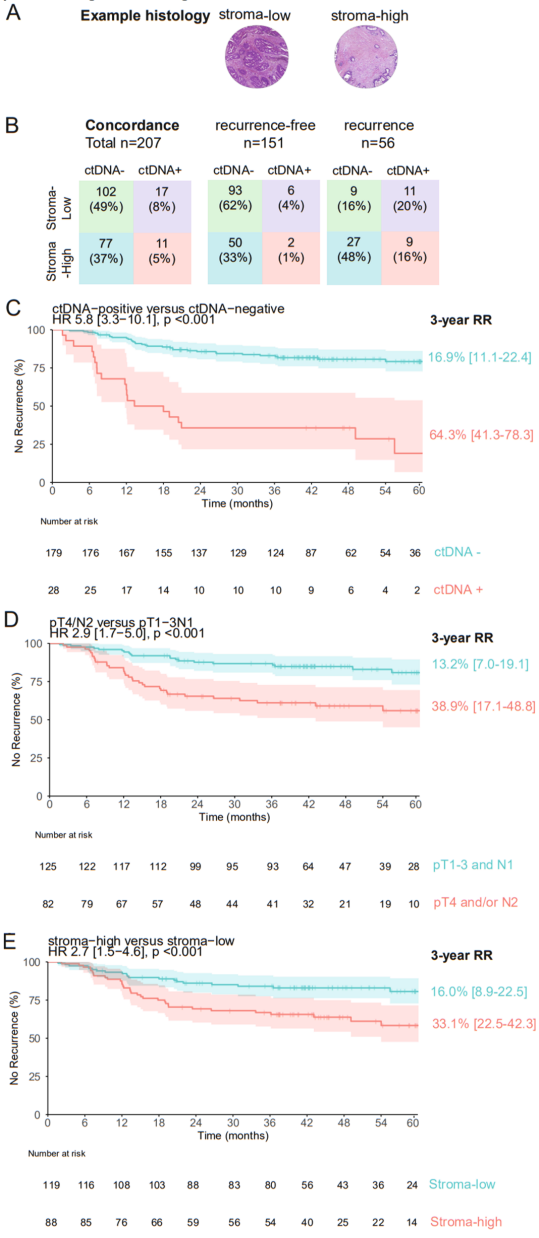
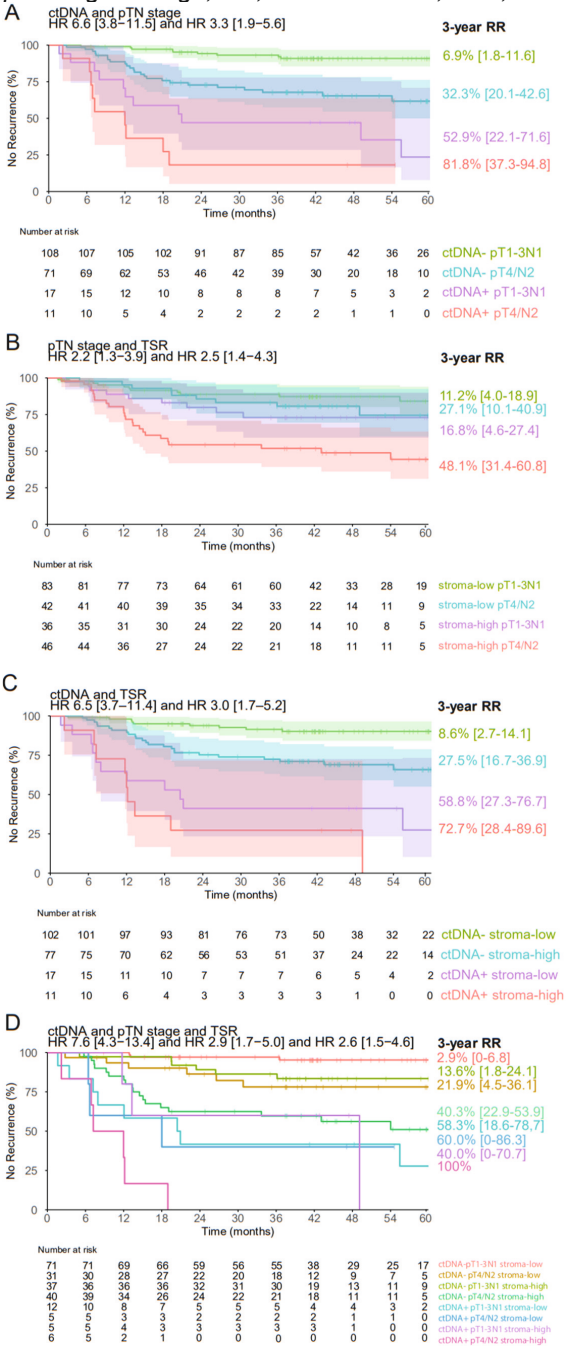
Supplementary material

Supplementary Figure 1: A) Example histology of TSR; B) Concordance with ctDNA, in the overall population and the subgroup with and without recurrence. Univariable time to recurrence analysis and reported recurrence rates of C) post-surgery ctDNA; D) pTN stage; E) TSR. *ctDNA, circulating tumor DNA; HR, hazard ratio; pTN, pathological stage; RR, recurrence rate; TSR, tumor-stroma ratio.*



Supplementary Figure 2: Time to recurrence analysis (no correction for other variables) of A) ctDNA and pTN stage; B) pTN stage and TSR; C) ctDNA and TSR. D) ctDNA, pTN stage and TSR. *ctDNA, circulating tumor DNA; HR, hazard ratio; pTN, pathological stage; RR, recurrence risk; TSR, tumor-stroma ratio.*



6

Cancer-associated fibroblast signature and SMAD4 mutation in resistance to adjuvant chemotherapy in stage III colon cancer patients

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Submitted

Abstract

Introduction

Stage III colon cancer (CC) is routinely treated with resection followed by adjuvant chemotherapy (ACT). However, 50% of patients are cured by surgical intervention alone, whilst another 30% experience disease recurrence despite ACT. This study aimed to identify biomarkers prognostic of recurrence and/or predictive of response to ACT.

Methods

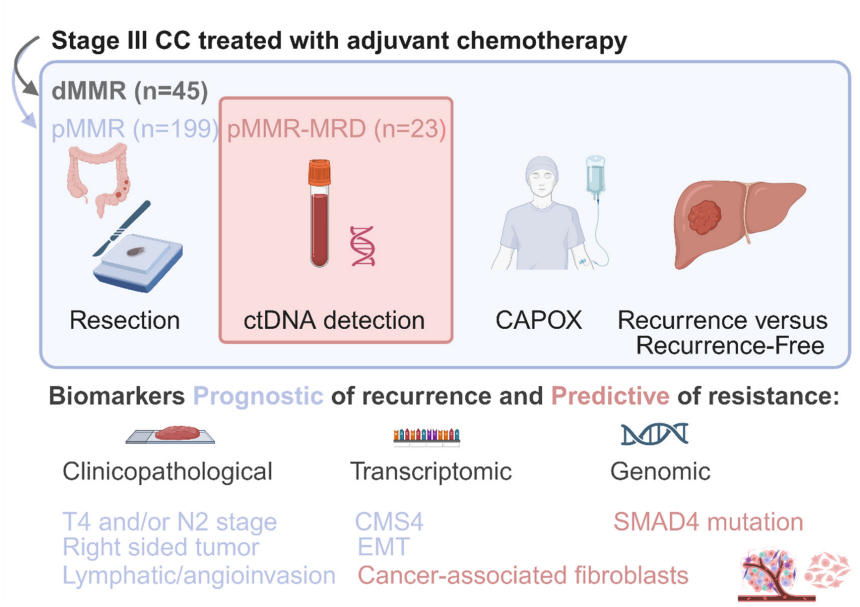
Prognostic value of clinicopathological features, transcriptional profiles and genomic mutations was examined for patients with microsatellite stable (MSS) and instable CC receiving ACT. Predictive value was examined in a sub-cohort of patients with minimal residual disease (MSS-MRD), defined by post-surgery circulating tumor DNA detection.

Results

In MSS patients (n=199), recurrence was associated with pT4 and/or pN2 tumors (HR 3.5 [2.0-5.9], p<0.001); CMS4 (HR 2.6 [95%CI 1.2-5.4], p=0.008); a high cancer-associated fibroblast (CAF) signature (HR 2.2 [1.4-3.6], p=0.001); and non-synonymous *SMAD4* mutations (HR 2.1 [1.1-4.2], p=0.027). In the MSS-MRD sub-cohort (n=23), lack of response to ACT was associated with a high CAF signature (HR 5.3 [1.7-17], p=0.002) and *SMAD4* mutations (HR 3.4 [0.9-13], p=0.060).

Discussion

A high CAF signature and *SMAD4* mutations have both prognostic value for recurrence and predictive value for response to ACT in stage III CC. This molecular profile provides leads to design novel therapies for patients resistant to standard ACT.


Stage III CC treated with adjuvant chemotherapy
dMMR (n=45)
pMMR (n=199)
pMMR-MRD (n=23)
Resection
ctDNA detection
CAPOX
Recurrence versus Recurrence-Free
Biomarkers Prognostic of recurrence and Predictive of resistance:
Clinicopathological
Transcriptomic
Genomic
T4 and/or N2 stage
Right sided tumor
Lymphatic/angioinvasion
CMS4
EMT
Cancer-associated fibroblasts
SMAD4 mutation

Introduction

The cornerstone of treatment of stage III colon cancer (CC) is resection, followed by adjuvant chemotherapy (ACT) with a fluoropyrimidine (5-fluorouracil (5-FU) or capecitabine) as single agent or combined with oxaliplatin (FOLFOX or CAPOX).[1,2] This one-size-fits-all approach has remained unchanged in the last two decades[3] and fails to cure the approximately 30% of patients who experience disease recurrence. Resection alone already cures half of patients.[4] Consequently, these patients are unnecessarily exposed to ACT and its associated toxicity.[5] The other half of patients have minimal residual disease (MRD) after surgery[4] and require systemic treatment. Recent studies show that MRD can be detected using circulating tumor DNA (ctDNA), which is indicative for the systemic presence of micrometastatic tumor cells following macroscopic resection of the primary tumor. Among patients with ctDNA-based MRD, ACT is unable to prevent recurrence in circa two-thirds of patients.[6-8] Hence, there is a need for prognostic biomarkers to identify patients at high risk of recurrence, as well as predictive biomarkers that predict lack of response to current ACT and inform personalized treatment.

Currently, pathological T stage (tumor extent) and N stage (lymph node involvement) are the most established risk factors to estimate risk and guide adjuvant treatment decisions. Stage III CC is defined by positive lymph nodes (pN+), with the pN2 and/or pT4 subset classified as high-risk stage III. Other prognostic factors include tumor location in the right hemicolon[9], no radical resection (R1 or R2), poor differentiation[10], lymphatic or vascular[11] or perineural[12] invasion, and tumor budding[13]. In addition to these epithelial characteristics, characteristics of the tumor microenvironment (TME) are also associated with a poor prognosis and chemoresistance[14], as shown for high stroma content[15] and a low level of immune cell infiltration[16]. More recently, deep learning analyses of complete histopathological slides, integrating tumor cell-intrinsic features and the TME, have demonstrated prognostic value.[17]

In addition to these histopathological markers, molecular biomarkers may carry prognostic value for recurrence and/or predictive value for benefit of treatment. For example, 15-20% of stage III CC has deficient mismatch repair (dMMR), resulting in microsatellite instability (MSI). Compared to proficient mismatch repair (pMMR)/ microsatellite stability (MSS), dMMR/MSI is associated with less recurrence[18,19], less

benefit from 5-FU[20] and oxaliplatin[21], and more sensitivity to immune checkpoint inhibition[22]. On the genomic level, both *BRAF* and *KRAS* mutations have been associated with resistance to standard chemotherapy.[23,24] On gene expression level, CC has been divided into four consensus molecular subtypes (CMS). CMS1 is associated with strong immune activation and *BRAF* mutation.[25] The epithelial CMS2 is characterized by chromosomal instability and best oxaliplatin response.[26] The smaller subset of metabolic CMS3 shows enriched *KRAS* mutation[25] and reduced CAP(OX) response[27]. The mesenchymal CMS4 displays stromal invasion low predicted benefit of adjuvant 5-FU.[28] While dMMR/MSI tumors mostly align with CMS1, the key value of CMS is in identifying the worse prognosis of CMS4 compared to CMS2 within the large and diverse pMMR/MSS subgroup.

In this study, we assessed the prognostic value of clinicopathological, transcriptomic and genomic biomarkers in stage III CC patients treated with ACT by comparing patients with versus without disease recurrence, stratified by MSS status. Moreover, based on post-surgery ctDNA detection, we defined a sub-cohort of MSS patients with MRD (MSS-MRD), for whom cure truly depends on response to ACT. This unique approach enables us to specifically evaluate which biomarkers have potential to predict who may (not) respond to ACT.

Methods

Study design

In total, 259 patients were selected from the Prospective Dutch CRC (PLCRC) cohort, which collects clinical data through the Netherlands Cancer Registry (NCR) and provides the infrastructure and written informed consent for the collection of resection tissue and blood samples (METC 12-510, NCT02070146).[29] Selection criteria were pathologically confirmed stage III CC, diagnosed in between 2016 and 2021, treated with adjuvant CAPOX or capecitabine, and no neoadjuvant treatment or prior malignancy in the past five years (Supplementary Figure 1).

RNA and DNA isolation

Formalin-fixed paraffin-embedded (FFPE) tumor blocks were collected through the Dutch Nationwide Pathology Databank (Palga)[30] and the tumor region was macro-dissected to isolate RNA and DNA, using the QIAGEN AllPrep RNA/DNA FFPE kit.

RNA sequencing and quality control

The Illumina TruSeq RNA Exome kit was used to produce RNA-seq libraries. RNA quality and quantity was assessed using the Agilent 2100 Bioanalyzer with the DNA 7500 chip. RNA-seq libraries were sequenced on a NovaSeq 6000 sequencing system (PE51bp). Raw reads were polyX and polyG trimmed using fastp (v0.23.2) at otherwise default settings and reads shorter than 20 nucleotides were discarded.[31] Expression of transcripts was quantified using salmon (v1.10.1) with full decoy-aware selective-alignment to the GRCh38 reference transcriptome (Ensembl release 107).[32] Samples with less than one million mapped fragments were excluded.

Because RNA-seq from FFPE requires quality control, we made a great effort in excluding samples with insufficient RNA-seq quality. The mean Pearson correlation of each sample to all other samples in the dataset was calculated. Next, *CIBRA* (v0.1.0)[33] was used to iteratively calculate the differential gene expression impact of known biologically relevant comparisons (e.g. patients with and without mutations in *APC* and *TP53*) and non-specific signal derived from 30 random permutations, after excluding samples at increasing thresholds of the calculated mean Pearson correlation (Supplementary Figure 2). Fourteen samples (5.4%) were excluded (Supplementary Figure 1), based on low RNA library complexity and gene coverage as determined using *RNAseqQC* (v0.1.4) (Supplementary Figure 2).[34]

RNA sequencing analyses

For the 245 samples of sufficient RNA-seq, a significant portion of the variance was primarily associated with RNA quality metrics before sequencing (DV200 and cDNA library prep concentration).[35] These metrics were therefore scaled, centered, and included as covariates in the final linear models for differential gene expression analysis between patients with and without recurrence: expression ~ DV200_scaled + cDNA_conc_scaled + Recurrence. Count normalization and subsequent analysis of differentially expressed genes (DEGs) was done using *DESeq2* (v1.44.0).[36] The lfcShrink function in the *apeglm* (v.1.22.1) package was used to shrink log2 fold changes.[37] Prior to further downstream processing, genes with low expression (in total <10 counts across samples) were filtered out. Counts were vst-normalized for subsequent principal component analysis. Genes were defined as significantly

differentially expressed at an absolute shrunken fold change >1.5 and p-value <0.05 after False Discovery Rate (FDR) correction using the Benjamin-Hochberg method. The CMS classifier's single sample predictor method (v1.0.0) was performed on the vst-normalized expression data.[25] To assess which cellular pathways were affected in the tested comparisons, we used the vst-normalized expression data to estimate enrichment scores for the 50 MSigDB hallmark gene sets[38] using gene set variation analysis (GSVA) (v1.48.3)[39]. Relative quality weights were estimated using the *limma* (v.3.56.2) arrayWeights function on the vst-normalized expression data with the design: DV200_scaled + cDNA_conc_scaled + Recurrence.[40] Pathways were considered significantly differentially enriched at FDR <0.05. The presence of immune cell subsets in the tumor microenvironment was deconvoluted using vst-normalized expression data (xCell) or gene-level transcript abundance (transcripts per million) (EPIC) as input.[41] The optimal cut-off fraction for the dichotomization of the abundance of immune cell subsets was determined using the *OptimalCutpoints* (v1.1-5) package with the Youden method and recurrence as the test variable.[42]

Protein expression of fibroblast markers

The tissue block used for RNA and DNA isolation was also used for further histological assessment. Tissue micro-arrays (TMAs; available for n=204) including 3 random cores per patient (0.6mm) were sectioned (4μM) for immunohistochemistry (IHC) on fibroblast activation protein (FAP ab207178 1:400) and alpha smooth muscle actin (αSMA ab5694 1:200). Slides were scanned using NanoZoomerXR (Hamamatsu) at 40x magnification, for quantification using QuPath's positive cell detection command.[43] A classifier was trained to segment tumor and stroma regions. Detected cells were classified as FAP/αSMA$^+$ or FAP/αSMA$^-$ based on the mean staining intensity. Per core, the proportion of FAP/αSMA$^+$ stromal and tumor cells was determined. Cores with at least 500 cell detections and visual good quality were used to average quantifications per patient.[44]

DNA sequencing analyses

DNA (available for n=240) was analyzed with a panel of 505 tumor-related genes using the PGDx® elio™ tissue complete assay.[45] Non-synonymous oncogenic variants were selected using OncoKb[46] and Clinvar[47]. Inconclusive variants were classified conform to the American College of Medical Genetics and Genomics guidelines.

MSS and MSI cohort

MMR/MSS status was determined based on DNA sequencing, except for four cases where mismatch repair proteins were evaluated using IHC. The one patient whose MMR/MSS status remained unknown was excluded, resulting in a total cohort of 244 patients (Supplementary Figure 1). Because of their distinct biology and high tumor mutational burden (TMB), the cohort of pMMR/MSS tumors (n=199, TMB<17 mut/Mb) was analyzed separately from the cohort of dMMR/MSI tumors (n=44, TMB>34 mut/Mb). One MSS tumor had a POLE mutation and a high TMB (535 mut/Mb). This hypermutator was expected to be biologically similar to MSI and was therefore assigned to the cohort further referred to as 'MSI' (Figure 1).

MSS-MRD sub-cohort

Blood samples (n=180) available at least four days after surgery and before initiation of ACT were used for ctDNA analyses (Labcorp® Plasma Detect™), as described in the PLCRC substudy PROVENC3.[8] Patients with detectable post-surgery ctDNA were selected as a sub-cohort of interest, because these patients were not cured by surgery alone and were expected to develop disease recurrence unless they were cured by response to ACT. Therefore, this MSS-MRD sub-cohort (n=23) was used to evaluate which biomarkers predict response to ACT (Figure 1). The small number of MSI-MRD patients (n=2) precluded meaningful analyses.

End points and statistical analysis

Analyses were performed separately in the MSS and MSI cohort for prognostic value for recurrence, and in the MSS-MRD sub-cohort for predictive value for response to ACT. The primary outcome measure was presence or absence of distant or local recurrence, as determined in standard clinical follow-up. Between patients with and without recurrence, clinicopathological characteristics and biomarkers were compared, using as appropriate χ^2 or Fisher's exact test for categorical variables and independent t-test or Mann-Whitney U for continuous variables. Time to recurrence (TTR) from date of surgery was compared across biomarkers in univariable log-rank and multivariable Cox proportional hazards models. Hazard ratios (HR) and 3-year recurrence risk (RR) were reported with 95% confidence intervals (CI), and median follow-up with interquartile range (IQR). Statistical analyses were performed using R statistical software (v4.2.2). Two-sided analyses with p-values <0.05 were considered significant.

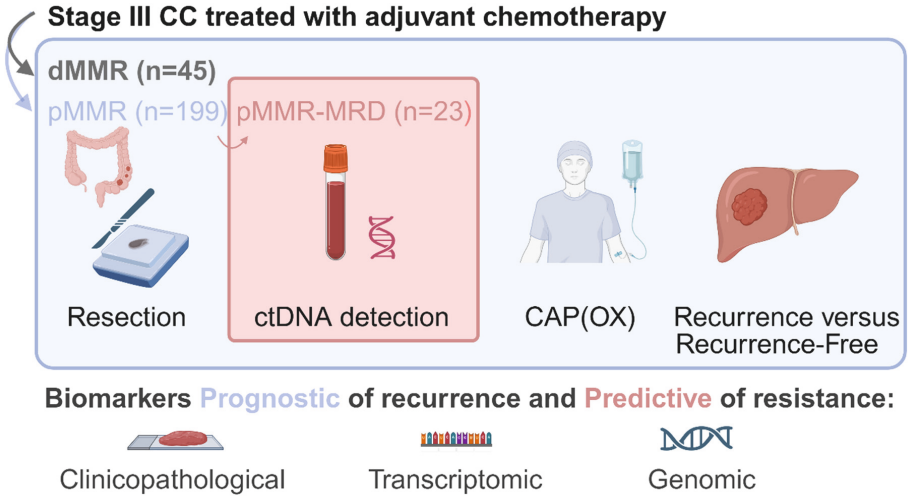


Figure 1: Stage III CC resection tissue was used to study clinicopathological, transcriptomic and genomic biomarkers, based on prognostic value for recurrence after resection and adjuvant CAP(OX) in the MSS and MSI cohort, as well as predictive value for response to adjuvant CAP(OX) in the MSS-MRD sub-cohort with MRD after resection based on ctDNA.*CC, colon cancer; ctDNA, circulating tumor DNA; MSI, microsatellite instable; MSS, microsatellite stable; MRD, minimal residual disease.*

Results

Conventional clinicopathological factors are prognostic of recurrence in MSS patients

Within the cohort of stage III CC patients who were treated with adjuvant CAP(OX) (Figure 1), we first aimed to identify prognostic and predictive features in the subset of MSS patients. To this end, patient and tumor characteristics were stratified by recurrence status (Table 1), in the total MSS cohort to assess prognostic value for recurrence and in the MSS-MRD sub-cohort to evaluate predictive value for response to ACT. Among the 199 MSS patients, 67 (34%) patients experienced recurrence (median TTR 14.5 months [IQR 9.2-29]) whilst 132 (66%) patients were recurrence-free for at least 3 years (median follow-up 52 months [IQR 41-61]). Prognostic factors associated with recurrence were clinicopathological high-risk based on pT4 and/or pN2 and lymphatic or angioinvasion. The 23 (14%) patients with MSS-MRD, defined by post-surgery ctDNA detection, had a higher RR (n=17 (74%)) and shorter TTR (11 months [IQR 7-13]), with preferential detection of metastases to the liver (Supplementary Table 1). Compared to the complete MSS cohort, the MSS-MRD sub-cohort had more advanced pT and pN stage, although this was not significantly associated with recurrence within MSS-MRD.

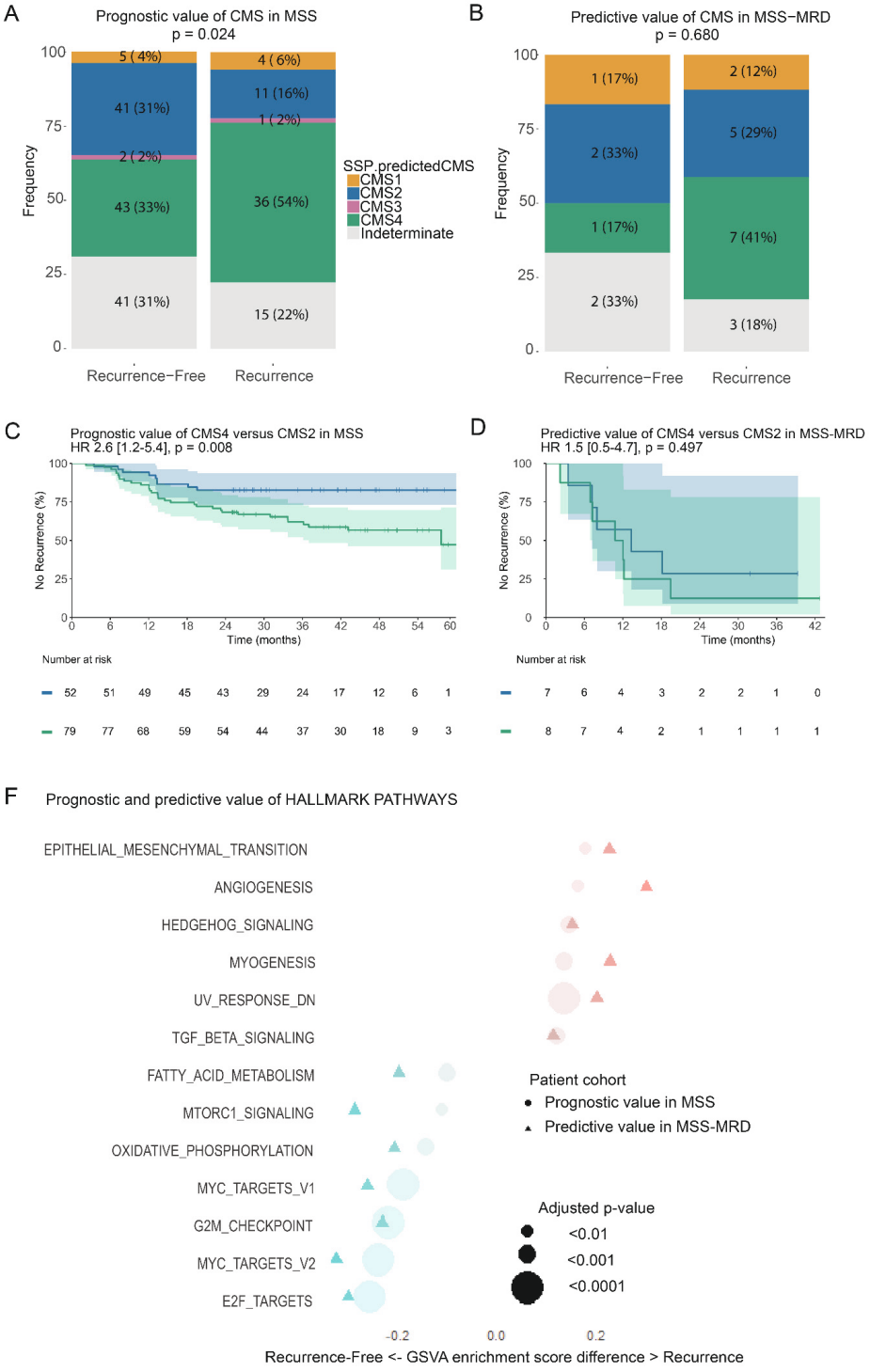
Table 1: Patient and tumor characteristics stratified by recurrence, per subgroup based on MSS and MSS-MRD.
AC, adenocarcinoma; MRD, minimal residual disease; MSS, microsatellite stable; SD, standard deviation.

	MSS Recurrence-Free (n=132)	Recurrence (n=67)	p-value	MSS-MRD Recurrence-Free (n=6)	Recurrence (n=17)	p-value
Age			0.390			0.260
Mean (SD)	62.2 (9.1)	63.5 (11)		60.5 (11)	67.0 (9.1)	
Sex			0.209			1
Female	57 (43%)	22 (33%)		2 (33%)	5 (29%)	
Male	75 (57%)	45 (67%)		4 (67%)	12 (71%)	
Clinical risk			<0.001			0.640
T1-3N1	93 (71%)	26 (35%)		4 (67%)	8 (47%)	
T4/N2	39 (29%)	41 (65%)		2 (33%)	9 (523%)	
T stage			<0.001			0.150
T1	3 (2.3%)	2 (3.0%)		0 (0%)	0 (0%)	
T2	27 (21%)	5 (7.5%)		1 (17%)	2 (12%)	
T3	83 (63%)	32 (48%)		5 (83%)	8 (47%)	
T4	19 (14%)	28 (42%)		0 (0%)	7 (41%)	
N stage			<0.001			1
N1	106 (80%)	36 (54%)		4 (67%)	10 (59%)	
N2	26 (20%)	31 (46%)		2 (33%)	7 (41%)	
Side			0.065			0.310
Left	90 (68%)	36 (54%)		4 (67%)	7 (41%)	
Right	42 (33%)	31 (46%)		2 (33%)	10 (59%)	
Histology			1			1
AC	118 (90%)	60 (90%)		6 (100%)	15 (88%)	
Other	14 (10%)	67(10%)		0 (0%)	2 (12%)	
Differentiation			0.184			0.539
Moderate	122 (93%)	56 (86%)		6 (100%)	14 (82%)	
Poor	9 (6.9%)	9 (14%)		0 (0%)	3 (18%)	
Missing	1	2		0	1	
Lymphatic or angioinvasion			<0.001			0.070
No	75 (60%)	17 (26%)		5 (83%)	5 (29%)	
Yes	50(40%)	48 (74%)		1 (17%)	12 (71%)	
Missing	7	1		0	0	
Radical resection			0.822			0.462
R0	126 (98%)	64 (98%)		5 (83%)	16 (94%)	
R1 or R2	3 (2%)	1 (2%)		1 (17%)	1 (6%)	
Missing	3	2		0	16	
Chemo			0.283			1
Capecitabine	10 (7.6%)	9 (13%)		1 (17%)	5 (29%)	
CAPOX	122 (92%)	58 (87%)		5 (83%)	12 (71%)	

CMS4 is prognostic for recurrence in MSS but not predictive in MSS-MRD sub-cohort
To evaluate the prognostic and predictive value of gene expression profiles, we performed bulk exome-RNA sequencing of the FFPE tumor resection tissue from the primary tumor. In the MSS cohort (n=199), 348 genes were significantly differentially expressed (fold change ≥1.5, p<0.05) between tumors derived from patients who experienced recurrence (n=67) versus those who remained recurrence-free (n=132) (Supplementary Figure 3; Supplementary Table 2-3). Patients with recurrence were more frequently CMS4 than those without recurrence (54% versus 33%, p=0.023), corresponding to the more mesenchymal consensus molecular subtype. Patients with CMS4 had a higher RR than CMS2 (HR 2.6 [95%CI 1.2-5.4], p=0.008), but this did not translate to a predictive value for response to ACT in the MSS-MRD cohort (n=23, HR 1.5 [0.5-4.7], p=0.497) (Figure 2A-D).

Prognostic and predictive value of gene sets related to 'hallmarks of cancer'
Next, we performed gene set variation analysis to investigate enrichment of hallmark pathways in patients with recurrence compared to patients without recurrence (all gene sets in Supplementary Table 4, significant results presented in Figure 2E). In the overall MSS cohort (n=199), the two gene sets with the largest difference between recurrence and recurrence-free patients were epithelial-mesenchymal transition (EMT; Difference=0.18, FDR=0.002) and angiogenesis (Difference=0.16, FDR=0.001). Comparable differences were found in the MSS-MRD cohort (n=23), but did not reach statistical significance (Difference=0.20, FDR=0.381; Difference=0.30, FDR=0.176). Conversely, tumors from patients who remained recurrence-free were enriched in gene sets linked to proliferation, identified by targets of E2F (MSS Difference=-0.26, FDR<0.001; MSS-MRD Difference=-0.30, FDR=0.182) and MYC (MSS Difference=-0.24, FDR<0.001; MSS-MRD Difference=-0.33, FDR=0.176) transcription factors.

Figure 2: Gene expression profile analysis between patients with versus without recurrence: Proportion of CMS1-4 in A) MSS and B) MSS-MRD (Fisher's exact test); TTR analysis in CMS4 versus CMS2 in C) MSS and D) MSS-MRD (log-rank test); E) GSVA enrichment score differences between patients with and without recurrence, showing significant results in MSS, along with the respective result in MSS-MRD.
CMS, consensus molecular subtype; GSVA, gene set variation analysis; HR, hazard ratio; MSS, microsatellite stable; MRD, minimal residual disease; SSP, single sample predictor; TTR, time to recurrence.


A
Prognostic value of CMS in MSS
p = 0.024
Frequency
100
75
50
25
0
5 (4%)
41 (31%)
2 (2%)
43 (33%)
41 (31%)
4 (6%)
11 (16%)
1 (2%)
36 (54%)
15 (22%)
Recurrence−Free
Recurrence
SSP.predictedCMS
CMS1
CMS2
CMS3
CMS4
Indeterminate
B
Predictive value of CMS in MSS−MRD
p = 0.680
1 (17%)
2 (33%)
1 (17%)
2 (33%)
2 (12%)
5 (29%)
7 (41%)
3 (18%)
C
Prognostic value of CMS4 versus CMS2 in MSS
HR 2.6 [1.2-5.4], p = 0.008
No Recurrence (%)
Time (months)
Number at risk
52 51 49 45 43 29 24 17 12 6 1
79 77 68 59 54 44 37 30 18 9 3
D
Predictive value of CMS4 versus CMS2 in MSS-MRD
HR 1.5 [0.5-4.7], p = 0.497
7 6 4 3 2 2 1 0
8 7 4 2 1 1 1 1
F
Prognostic and predictive value of HALLMARK PATHWAYS
EPITHELIAL_MESENCHYMAL_TRANSITION
ANGIOGENESIS
HEDGEHOG_SIGNALING
MYOGENESIS
UV_RESPONSE_DN
TGF_BETA_SIGNALING
FATTY_ACID_METABOLISM
MTORC1_SIGNALING
OXIDATIVE_PHOSPHORYLATION
MYC_TARGETS_V1
G2M_CHECKPOINT
MYC_TARGETS_V2
E2F_TARGETS
-0.2
0.0
0.2
Recurrence-Free <- GSVA enrichment score difference > Recurrence
Patient cohort
Prognostic value in MSS
Predictive value in MSS-MRD
Adjusted p-value
<0.01
<0.001
<0.0001

Prognostic and predictive value of cancer-associated fibroblast signature

To gain insight into the type of cells associated with poor prognosis or treatment resistance, we used the bulk gene expression data to deconvolute cellular subsets of the tumor microenvironment and determine immune and stromal phenotypes based on EPIC and xCell (Supplementary Table 5). In the MSS cohort (n=199), patients with recurrence (n=67) showed a significantly higher fraction of cancer-associated fibroblast (CAFs) based on EPIC (p=0.016), and CAFs (p=0.032) and stroma score (p=0.023) based on xCell. Recurrence was also associated with gene signatures indicative of higher M2 macrophages (p=0.016) and lower B cell plasma cells (p=0.001) and T cells (various subtypes, Supplementary Figure 4). An EPIC-determined CAF fraction of 0.34 was the optimal cut-off to distinguish MSS patients with versus without recurrence. Using this cut-off, 80 patients (40%) were classified as CAF-high and showed a worse prognosis (3-year RR 41% [29-51]) compared to the 119 patients classified as CAF-low (3-year RR 18% [11-25]) (HR 2.2 [1.4-3.6], p=0.001) (Figure 3A, Table 2).

In line with the poor prognostic value of a high CAF signature within the MSS cohort, in the MSS-MRD sub-cohort (n=23) more patients were classified as CAF-high (52%). Moreover, the CAF signature was highly predictive of recurrence and thus resistance to ACT in MSS-MRD (HR 5.3 [1.7-17], p=0.002) (Figure 3B). Of the 12 patients classified as CAF-high, 11 (92%) developed a recurrence despite ACT. Of the 6 MSS-MRD patients who remained recurrence-free, 5 were CAF-low (71%). The other cell types deconvoluted using EPIC or xCell, including M2 macrophages and B cells and T cells, were not significantly different between patients with and without recurrence and thus not predictive of response in MSS-MRD (Supplementary Figure 4B).

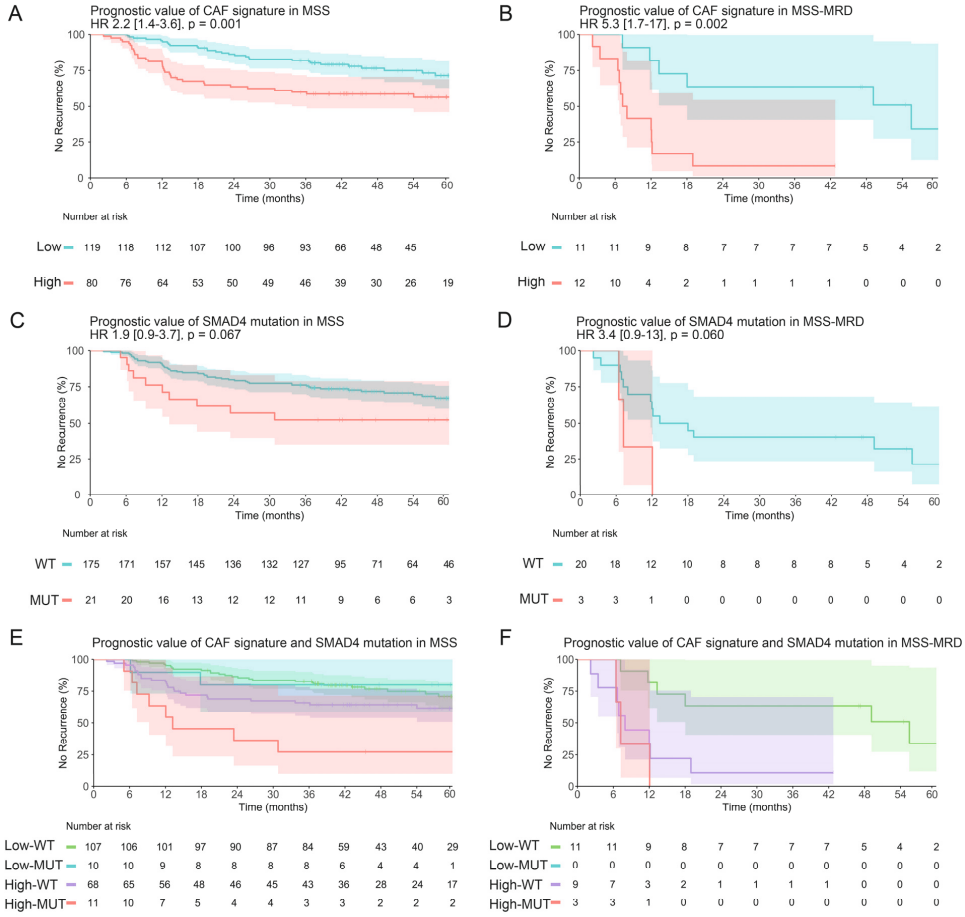


Figure 3: TTR analysis of the main biomarkers with prognostic value in MSS and predictive value in MSS-MRD: A-B) Immune cell deconvolution-based CAF signature, classified as High (>0.34) versus Low; C-D) Targeted DNA sequencing-based *SMAD4* mutant versus wild type; E-F) Combination of CAF signature and *SMAD4* mutation. *CAF, cancer-associated fibroblast; HR, hazard ratio; MRD, minimal residual disease; MSS, microsatellite stable; MUT, mutant; WT, wild type*.

The predictive value of the CAF signature is not captured by IHC on FAP or αSMA

The CAF signature consists of multiple individual genes with significant prognostic value in MSS patients and a similar trend in MSS-MRD patients (Supplementary Figure 5A-B). An example is *LUM* (Lumican; p=0.007), a mesenchymal marker in both the CAF signature and EMT signature, which is reported to be expressed by both stromal cells and neoplastic cells. To delineate whether the predictive impact of CAFs was mostly attributed to expression by stromal cells or mesenchymal neoplastic cells, the CAF marker *FAP* (RNA p=0.013) and myofibroblast marker *αSMA* (RNA p<0.001) were assessed using IHC on TMAs from the resected tumor specimen. Both markers were predominantly expressed in stromal cells and not in neoplastic cells. The median proportion of FAP+ (61% versus 61%, p=0.593) and αSMA+ (92% versus 92%, p=0.941) stromal cells did not differ between patients with versus without recurrence in MSS, nor in MSS-MRD patients (Supplementary Figure 6). These findings indicate that protein expression of these individual fibroblast markers was not able to capture the same prognostic and predictive value as the CAF signature.

Prognostic and predictive value of SMAD4 mutations

Tumor-specific mutations in a panel of 505 cancer-related genes were compared between patients with and without recurrence (Supplementary Table 7-8; the 20 most prevalent are presented in Figure 4). In the overall MSS cohort (n=196), known CC driver mutations were observed at representative rates described in prior studies. *KRAS* and $BRAF^{V600E}$ mutations have been described to be prognostic and potentially predictive for response to chemotherapy and constitute targets for alternative targeted therapy. No differences in mutation rates were observed between patients with versus without recurrence in *KRAS* (49% versus 47%, p=0.765) based on the Fisher's exact test. $BRAF^{V600E}$ mutation was numerically but not significantly enriched in MSS patients with versus without recurrence (14% versus 7%, p=0.079), but not in MSS-MRD patients (n=23, 12% versus 17%, p=1). *SMAD4* showed the most pronounced numeric difference in mutation rate between patients with and without recurrence in MSS (15% versus 8%, p=0.148) and MSS-MRD patients (18% versus 0%, p=0.539). In TTR analyses, *SMAD4* mutation was the only genomic marker with significant prognostic value in MSS (HR 2.1 [1.1-4.2], p=0.031) and potentially predictive value in the smaller MSS-MRD cohort (HR 3.4 [0.9-13], p=0.060) (Figure 3C-D).

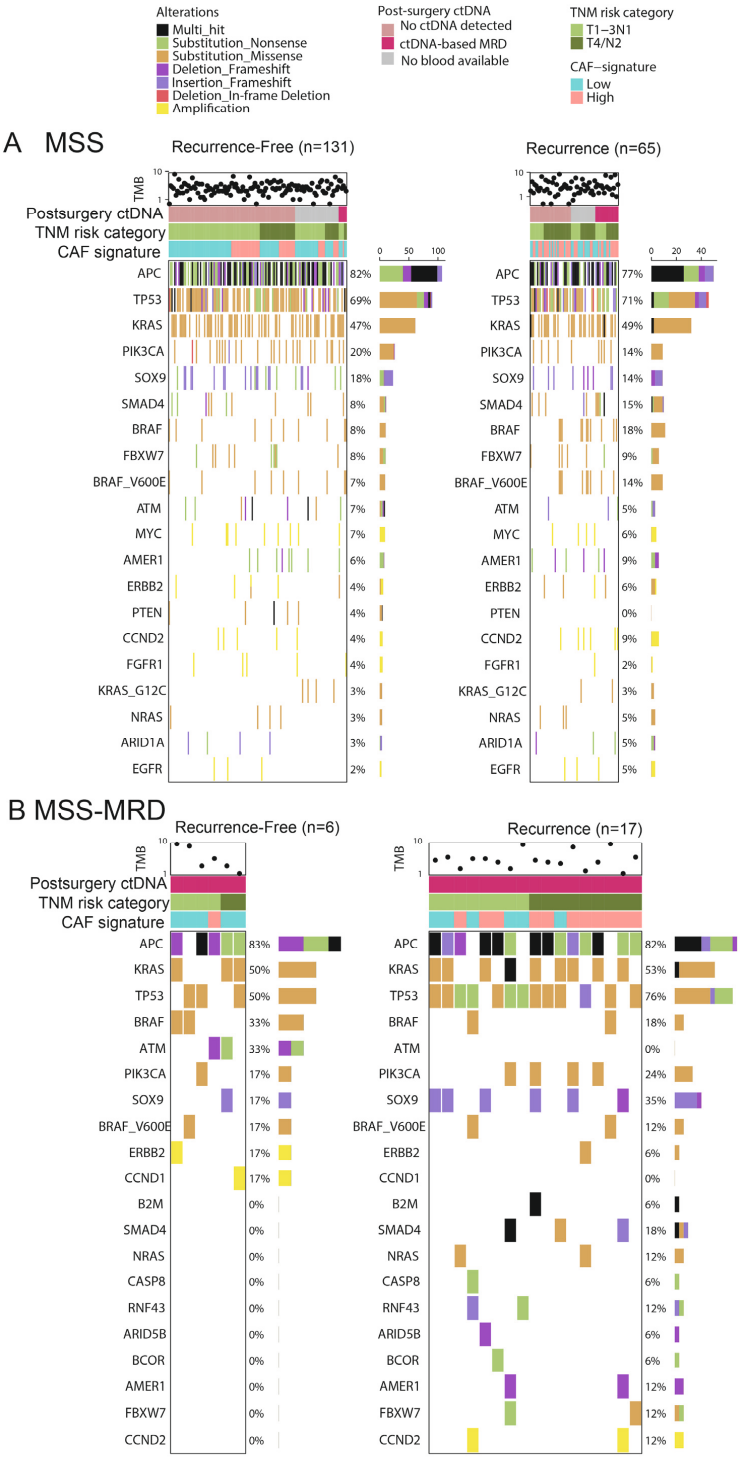

Alterations
Multi_hit
Substitution_Nonsense
Substitution_Missense
Deletion_Frameshift
Insertion_Frameshift
Deletion_In-frame Deletion
Amplification
Post-surgery ctDNA
No ctDNA detected
ctDNA-based MRD
No blood available
TNM risk category
T1–3N1
T4/N2
CAF–signature
Low
High
A MSS
Recurrence-Free (n=131)
Recurrence (n=65)
TMB
Postsurgery ctDNA
TNM risk category
CAF signature
APC 82% 77%
TP53 69% 71%
KRAS 47% 49%
PIK3CA 20% 14%
SOX9 18% 14%
SMAD4 8% 15%
BRAF 8% 18%
FBXW7 8% 9%
BRAF_V600E 7% 14%
ATM 7% 5%
MYC 7% 6%
AMER1 6% 9%
ERBB2 4% 6%
PTEN 4% 0%
CCND2 4% 9%
FGFR1 4% 2%
KRAS_G12C 3% 3%
NRAS 3% 5%
ARID1A 3% 5%
EGFR 2% 5%
B MSS-MRD
Recurrence-Free (n=6)
Recurrence (n=17)
TMB
Postsurgery ctDNA
TNM risk category
CAF signature
APC 83% 82%
KRAS 50% 53%
TP53 50% 76%
BRAF 33% 18%
ATM 33% 0%
PIK3CA 17% 24%
SOX9 17% 35%
BRAF_V600E 17% 12%
ERBB2 17% 6%
CCND1 17% 0%
B2M 0% 6%
SMAD4 0% 18%
NRAS 0% 12%
CASP8 0% 6%
RNF43 0% 12%
ARID5B 0% 6%
BCOR 0% 6%
AMER1 0% 12%
FBXW7 0% 12%
CCND2 0% 12%

Figure 4: Genomic alterations present in the top 20 most frequently affected genes in targeted DNA sequencing of 505 cancer-related genes in A) MSS and B) MSS-MRD. Patients are clustered by recurrence status, circulating tumor DNA status and TNM-based risk category. *CAF, cancer-associated fibroblast; ctDNA, circulating tumor DNA; MRD, minimal residual disease; MSS, microsatellite stable; TMB, tumor mutational burden; TNM, tumor node metastasis.*

Integration of clinicopathological biomarkers, CAF signature and SMAD4 mutation

We next investigated whether the CAF signature and *SMAD4* mutation, both involved in TGF-β signaling, exert an overlapping or a synergistic prognostic effect. *SMAD4* mutation was observed in 14% of CAF-high and 8.5% of CAF-low MSS tumors. In MSS, the high CAF signature (HR 2.2 [1.3-3.6], p=0.003) and presence of *SMAD4* mutations (HR 2.0 [1.1-4.0], p=0.042) had independent prognostic value when combined in a multivariable Cox model (Figure 3E). This remained true when CAF signature (HR 1.9 [1.1-3.2], p=0.024) and *SMAD4* mutation (HR 2.8 [1.3-4.7], p=0.006) were added to a Cox model with the clinicopathological risk factors pT4/N2 stage (HR 3.2 [1.8-5.5], p<0.001), right tumor sidedness (HR 2.1 [1.2-3.8], p=0.022) and lymphatic or angioinvasion (HR 3.0 [1.6-5.6], p<0.001) (Table 2).

In the MSS-MRD cohort, CAF signature and *SMAD4* mutation were the only predictors with a higher univariable HR compared with in the MSS cohort (Table 2), suggesting a specific role in chemoresistance. A multivariable model to combine predictors was not powered due to the small sample size of the MSS-MRD cohort. However, it was evident that all patients with *SMAD4* mutant tumors (n=3) were also CAF-high and experienced recurrence within a year (Figure 3F). In contrast, all six recurrence-free patients had *SMAD4* wild-type tumors (n=6) and nearly all were also CAF-low (n=5).

Table 2: Clinicopathological, genomic and transcriptomic risk factors in a Cox model. *HR, hazard ratio; MSS, microsatellite stable; *NA: not assessed due to small sample*.

	MSS multivariable		**MSS univariable**		**MSS-MRD univar**		**MSI univar**	
	HR [95%CI]	**p-value**	**HR [95%CI]**	**p-value**	**HR [95%CI]**	**p-value**	**HR [95%CI]**	**p-value**
Clinical risk T4/N2	3.2 [1.8-5.5]	<0.001	3.0[1.8-4.8]	<0.001	2.0 [0.7-5.3]	0.178	9.8 [1.1-84]	0.011
Tumor sidedness right	2.1 [1.2-3.8]	0.022	1.6 [1.0–2.6]	0.046	1.5 [0.6-4.1]	0.388	1.5 [0.2-13]	0.722
Lymph/angioinvasion yes	3.0 [1.6-5.6]	<0.001	3.1 [1.8-5.3]	<0.001	2.2 [0.8-6.4]	0.129	1.5 [0.3-8.3]	0.632
CAF fraction >0.34	1.9 [1.1-3.1]	0.024	2.2 [1.4-3.6]	0.001	5.3 [1.7-17]	0.002	4.2 [0.8-23]	0.074
SMAD4 mutant	2.8 [1.3-5.8]	0.006	2.1 [1.1-4.2]	0.027	3.4 [0.9-13]	0.060	3.8 [0.5-33]	0.186
BRAFV600E mutant	1.2 [0.5-3.3]	0.686	1.8 [0.9-3.5]	0.092	1.7 [0.4-7.6]	0.485	1.1 [0.2-5.9]	0.934
KRAS mutant	1.1 [0.6-2.1]	0.820	1.2 [0.8-2.0]	0.401	1.1 [0.4-2.7]	0.916	NA*	

High CAF signature and low T cell signature are also prognostic in the MSI cohort

Finally, we investigated whether the biomarkers were also prognostic in the smaller cohort of hypermutant tumors based on dMMR/MSI (n=44) or POLE mutation (n=1). Only 6 patients (14%) experienced a recurrence (TTR 7 months [6-9]) and 39 patients remained recurrence-free (median follow-up of 45 months [IQR 41-60]). Compared to MSS patients, MSI patients were more often female with right-sided tumors (Supplementary Table 1). Within the MSI cohort, only pN2 stage (p=0.003) and clinicopathological high-risk based on pT4 and/or pN2 (p=0.023) were significantly enriched in MSI patients with versus without recurrence (Supplementary Table 9).

RNA sequencing showed 271 differentially expressed genes between MSI patients with and without recurrence (Figure 5A; Supplementary Table 10). Most MSI tumors were classified as CMS1 (67% versus 71% in patients with versus without recurrence, p=0.443) (Figure 5B). Pathway analysis showed similar enrichment for EMT in recurrence as in MSS, but did not reach statistical significance in this small cohort (Difference=0.20, p=0.256) (Figure 5C; Supplementary Table 4). As for immune cell deconvolution, the gene signature associated with CAFs were most notably enriched among patients with recurrence (xCell p=0.097; EPIC p=0.034), while CD4 memory T cells were reduced (xCell p=0.005) (Figure 5D; Supplementary Table 5). Using the cut-off of 0.34, a high CAF signature was observed in 4 of 6 (67%) recurring patients and 12 of 37 (32%) recurrence-free patients. CAF-high patients showed a trend to worse recurrence risk (3-year RR 27% [1-46]) compared to patients CAF-low patients (3-year RR 7% [0-51], HR 4.2 [0.8-24, p=0.074) (Figure 5E; Table 2). The prognostic value of the CAF signature was not captured by FAP or αSMA protein expression in the stromal or epithelial compartment of the tumor (Supplementary Table 6).

The mutational profile for MSI tumors showed a high TMB and *BRAF*^{V600E} mutation rate, namely 67% in both MSI patients with and without recurrence. *SMAD4* was mutated in two patients, one of which experienced disease recurrence (17% versus 3%, p=0.262). Another gene involved in TGF-β signaling, *TGFBR2*, was mutated in 33% of patients with recurrence versus 57% of patients without recurrence (p=0.393). Of the seven patients with both a high CAF signature and wild type *TGFBR2*, three experienced recurrence, compared to one of the 15 of the CAF-low *TGFBR2* mutant patients (Figure 5F). Of the six patients with recurrence, five were either CAF-high (n=4) or *TGFBR2* wild type (n=4). No other significant differences in the cancer-related genes were observed across recurrence status (Figure 5G; Supplementary Table 7-8).

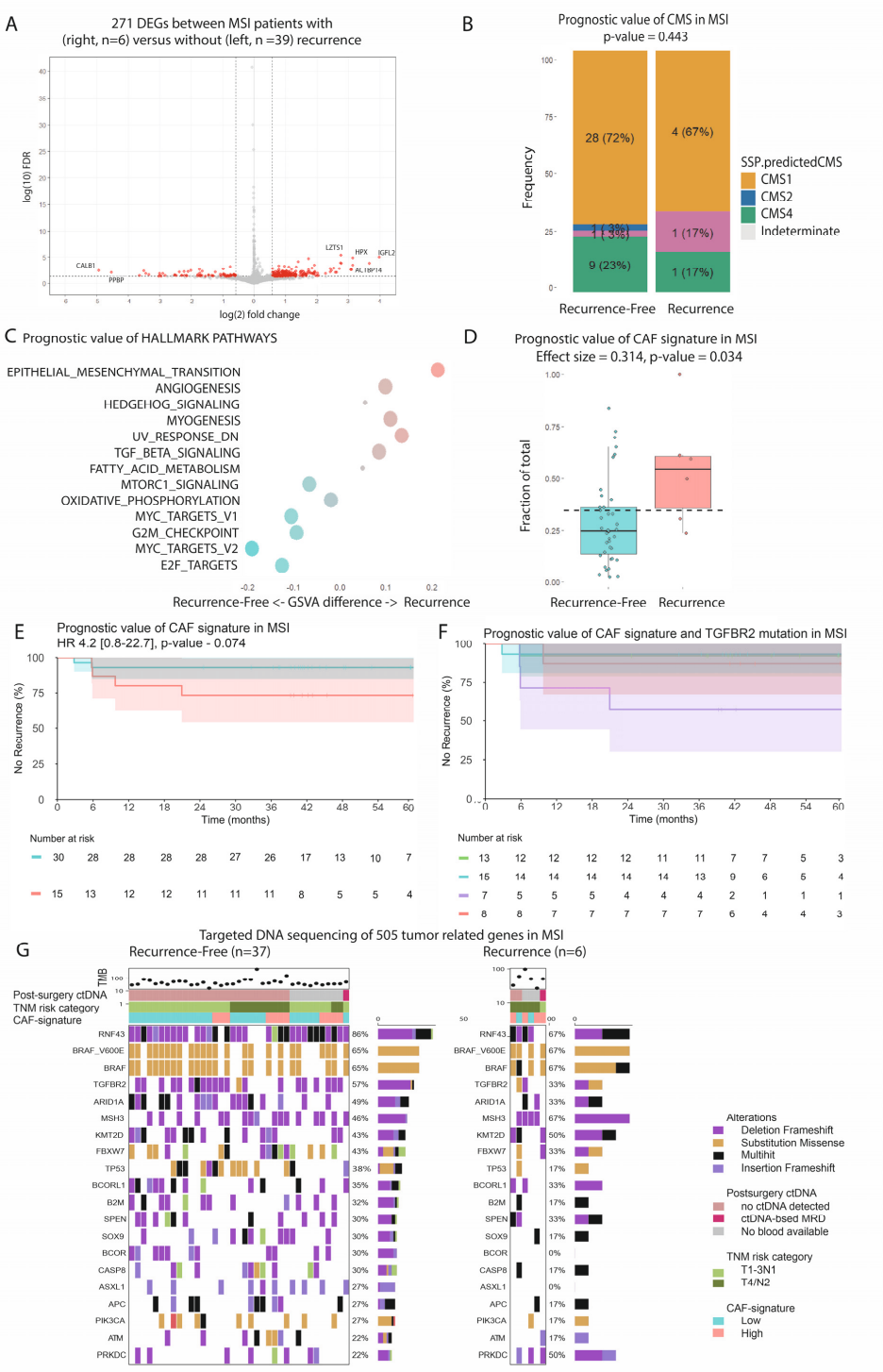

A
271 DEGs between MSI patients with
(right, n=6) versus without (left, n =39) recurrence
log(10) FDR
log(2) fold change
CALB1
PPBP
LZTS1
HPX
IGFL2
AL189714
B
Prognostic value of CMS in MSI
p-value = 0.443
Frequency
28 (72%)
4 (67%)
1 (3%)
1 (17%)
9 (23%)
1 (17%)
SSP.predictedCMS
CMS1
CMS2
CMS4
Indeterminate
Recurrence-Free
Recurrence
C
Prognostic value of HALLMARK PATHWAYS
EPITHELIAL_MESENCHYMAL_TRANSITION
ANGIOGENESIS
HEDGEHOG_SIGNALING
MYOGENESIS
UV_RESPONSE_DN
TGF_BETA_SIGNALING
FATTY_ACID_METABOLISM
MTORC1_SIGNALING
OXIDATIVE_PHOSPHORYLATION
MYC_TARGETS_V1
G2M_CHECKPOINT
MYC_TARGETS_V2
E2F_TARGETS
Recurrence-Free <- GSVA difference -> Recurrence
D
Prognostic value of CAF signature in MSI
Effect size = 0.314, p-value = 0.034
Fraction of total
Recurrence-Free
Recurrence
E
Prognostic value of CAF signature in MSI
HR 4.2 [0.8-22.7], p-value - 0.074
No Recurrence (%)
Time (months)
Number at risk
30 28 28 28 28 27 26 17 13 10 7
15 13 12 12 11 11 11 8 5 5 4
F
Prognostic value of CAF signature and TGFBR2 mutation in MSI
No Recurrence (%)
Time (months)
Number at risk
13 12 12 12 12 11 11 7 7 5 3
15 14 14 14 14 14 13 9 6 5 4
7 5 5 5 4 4 4 2 1 1 1
8 8 7 7 7 7 7 6 4 4 3
G
Targeted DNA sequencing of 505 tumor related genes in MSI
Recurrence-Free (n=37)
Recurrence (n=6)
TMB
Post-surgery ctDNA
TNM risk category
CAF-signature
RNF43 86% 67%
BRAF_V600E 65% 67%
BRAF 65% 67%
TGFBR2 57% 33%
ARID1A 49% 33%
MSH3 46% 67%
KMT2D 43% 50%
FBXW7 43% 33%
TP53 38% 17%
BCORL1 35% 33%
B2M 32% 17%
SPEN 30% 33%
SOX9 30% 17%
BCOR 30% 0%
CASP8 30% 17%
ASXL1 27% 0%
APC 27% 17%
PIK3CA 27% 17%
ATM 22% 17%
PRKDC 22% 50%
Alterations
Deletion Frameshift
Substitution Missense
Multihit
Insertion Frameshift
Postsurgery ctDNA
no ctDNA detected
ctDNA-bsed MRD
No blood available
TNM risk category
T1-3N1
T4/N2
CAF-signature
Low
High

Figure 5: Prognostic value of biomarkers in the MSI cohort: A) Differential gene expression between patients with (right) versus without recurrence (left); B) Consensus molecular subtype per recurrence status; C) GSVA on Hallmark gene sets between patients with (right) versus without recurrence (left); D) CAF signature based on EPIC, E) TTR analysis for CAF-high (>0.34) versus CAF-low tumors; F) TTR analysis for CAF signature and *TGFBR2* mutation; G) Genomic alterations in the top 20 most frequently affected genes in targeted DNA sequencing of 505 cancer-related genes.
CMS, consensus molecular subtype; ctDNA, circulating tumor DNA; GSVA, gene set variation analysis; HR, hazard ratio; MSI, microsatellite instable.

Discussion

This study aimed to identify clinicopathological, transcriptomic and genomics biomarkers associated with recurrence and chemoresistance in stage III CC patients receiving standard adjuvant CAP(OX). Clinicopathological high-risk (pT4 and/or N2), CMS4 and epithelial mesenchymal transition were prognostic of recurrence. A high CAF signature and *SMAD4* mutations were the only markers with next to prognostic value also a significant predictive value for resistance to adjuvant CAP(OX) in the sub-cohort of MSS patients with ctDNA-defined MRD after surgery.

In the MSS cohort, we observed a recurrence rate of ~30% after adjuvant CAP(OX), consistent with other studies.[4] Recurrence was associated with conventional clinicopathological risk factors indicating locoregional extent, including pT4 and/or pN2 stage, lymphatic or angioinvasion and tumor sidedness. Gene expression signatures related to the composition of the tumor microenvironment, like CMS4 and EMT and the CAF signature, were prognostic of recurrence. By focusing on the MSS-MRD sub-cohort of patients, i.e. the subset of patients who were not cured by surgery alone, we could search for biomarkers that are predictive of resistance to chemotherapy and identified the CAF signature and *SMAD4* mutations. Interestingly, both CAFs and *SMAD4* are involved in processes associated with shaping the tumor microenvironment.[48] *SMAD4* transduces TGF-β signaling between CAFs and tumor cells, subsequently playing a role in reinforcing CMS4 traits[49], remodeling the extracellular matrix, shaping an immunosuppressive TME, and promoting angiogenesis.[50] *SMAD4* mutation[51] or loss[52] in colon cancer has been associated with a worse prognosis as well as with fluoropyrimidine resistance[53]. Despite their functional relatedness, CAF signature and *SMAD4* mutations had added value in a Cox model.

The patients with MSI tumors were analyzed separately because of the distinct biological behavior, lower recurrence rate (13%) and lower response to chemotherapy.[20] Due to the limited number of patients and recurrences, we were not able to perform multivariable analysis or an MSI-MRD sub-cohort analysis. Nevertheless, like for the MSS cohort, we could demonstrate prognostic value of the CAF signature. Almost all MSI patients with a recurrence had a CAF-high signature and/or absence of inactivating mutation in *TGFBR2*. The transforming growth factor β receptor, upstream of the TGF-β signaling pathway, is prone to inactivating mutations in MSI CC, which have been associated with reduced EMT and reduced immune evasion and reduced metastatic potential.[54]

Bulk RNA sequencing does not provide information regarding whether the CAF signature is expressed by stromal cells in the TME, by mesenchymal neoplastic cells or both. Although we confirmed prognostic value of the established CAF marker FAP on RNA expression level, consistent with other studies[14,55], we could not confirm this finding by immunohistochemical evaluation of FAP protein expression in either the stromal or neoplastic compartment. This may be due to discrepancies between RNA and protein expression or the CAF signature captures a broader phenotype that cannot be reduced to solely stromal markers. Therefore, it may be challenging to capture the multi-gene RNA-based CAF signature in a single protein immunohistochemical assay, which would facilitate its implementation in routine diagnostic workflows.

Since patients with stage III CC with a CAF-high signature and genomic alterations in the TGF-β signaling pathway (mutant *SMAD4* in MSS, wild type *TGFBR2* in MSI) showed limited response to standard adjuvant CAP(OX), future studies should focus on developing alternative treatment strategies for this group of chemotherapy-resistant tumors. In patients with metastatic CC, intrinsic resistance to 5-FU has been associated with *SMAD4* mutations and has been restored by adding the STAT3 inhibitor GB201.[56] Other potential therapeutic targets are the signaling pathways involved in maintaining a mesenchymal tumor cell state and recruiting CAFs, including transforming growth factor β (TGFβ; LY3200882/galunisertib[57]) or fibroblast growth factor (FGF; pemigatinib[58]) or platelet-derived growth factor (PDGF; imatinib[59]). Studies attempting to target CAFs with car-T cells[60], antibodies (sibrotuzumab[61]) and small molecules (talabostat[62]) have not been proven successful yet in CC.

The unique strength of our study lies in the ability to evaluate the predictive value of biomarkers for response to ACT. In general, this is a challenge in adjuvant studies, as recurrence of disease may be prevented already by the surgical resection alone and therefore does not reflect sensitivity to chemotherapy. Advances in post-surgery ctDNA testing allowed us to identify a sub-cohort of patients with MRD, who were not cured by surgery and depended on chemosensitivity for cure. Hence, a ctDNA-based definition of patients with MRD provides the clinically relevant opportunity to classify patients as resistant or sensitive to the ACT. By further characterizing this cohort on transcriptomic and genomic markers in a multiomic approach, we were able to explore the mechanisms associated with resistance to chemotherapy. This may ultimately facilitate the development of predictive biomarkers and novel treatment strategies tailored to the identified ACT-resistant population. Although ctDNA detection is the strongest prognostic biomarker and measure of MRD to date[6,8,63,64] the current test is especially effective at detecting micrometastases to the liver, while the lung and peritoneum remain more challenging[8,65]. As a result, these low ctDNA-shedding sites are underrepresented in our MRD cohort. Thus, the predictive value of the CAF signature and *SMAD4* mutations may primarily apply to liver metastases and may not be generalizable to all metastatic sites.

In conclusion, in patients with stage III CC a CAF-high gene signature was associated with an increased risk of recurrence due to resistance to adjuvant CAP(OX). In the MSS subgroup, other mesenchymal signatures like CMS4 and EMT were prognostic of recurrence, but not predictive of response to adjuvant CAP(OX) in patients with MRD. Moreover, on a genomic level *SMAD4* mutations had predictive value and added prognostic value to the CAF signature and conventional clinicopathological risk factors. Taken together, our data highlight that these mesenchymal features of the tumor and microenvironment may be used to stratify patients who are resistant to standard-of-care ACT. This may provide avenues for the development of novel therapeutic interventions to specifically target this subgroup of CAP(OX)-resistant patients.

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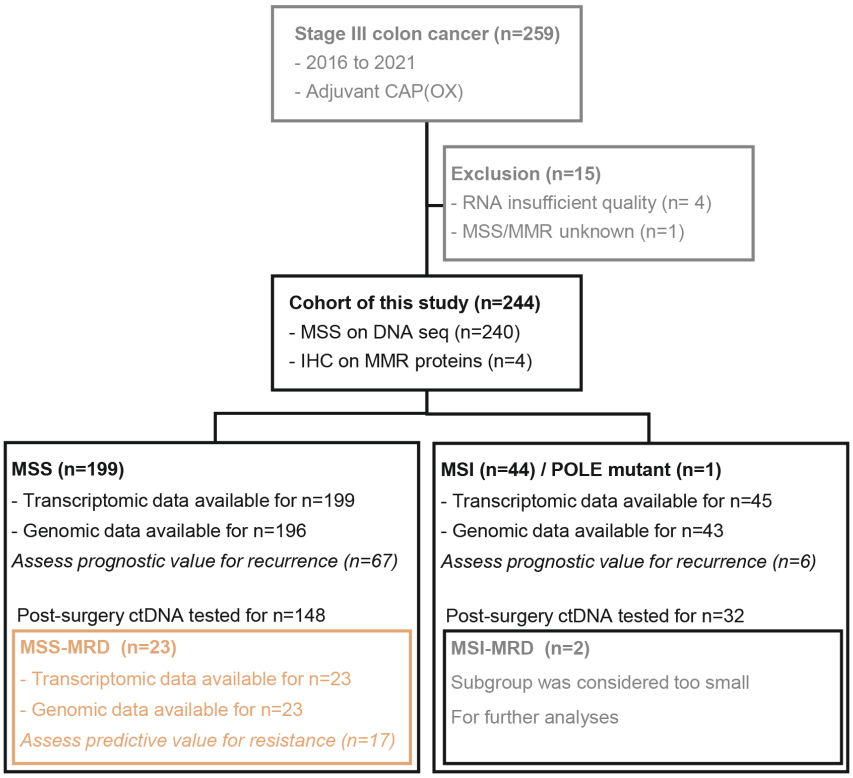
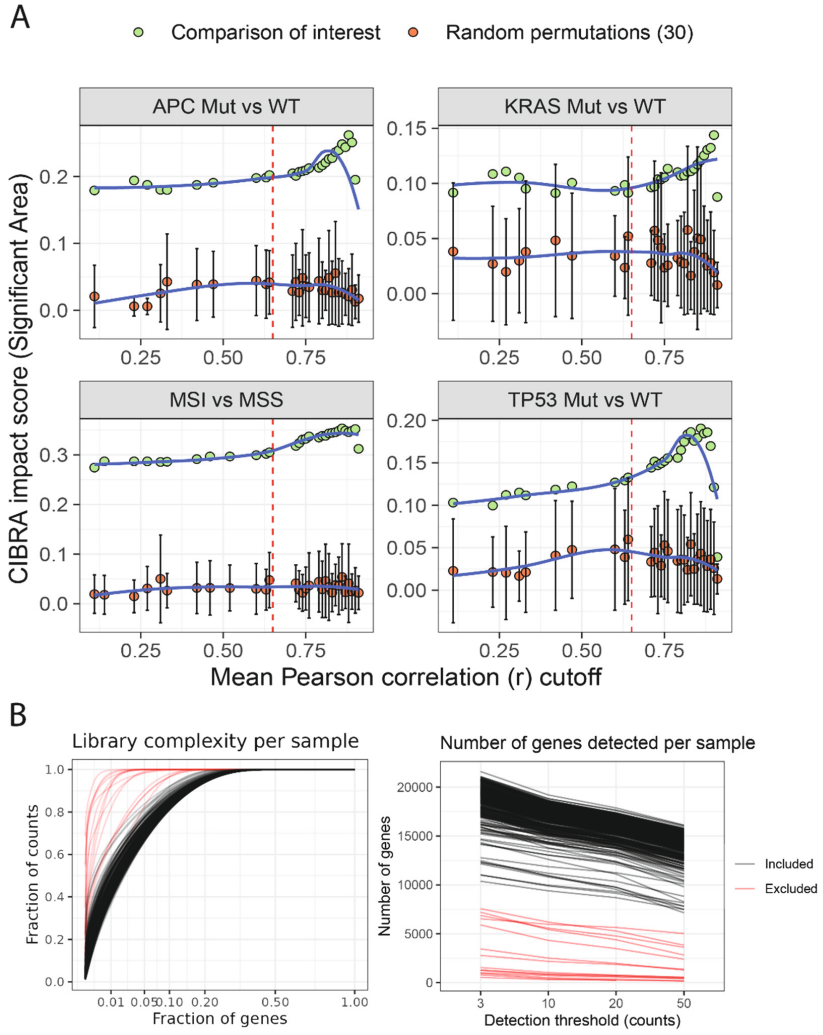
Supplementary material

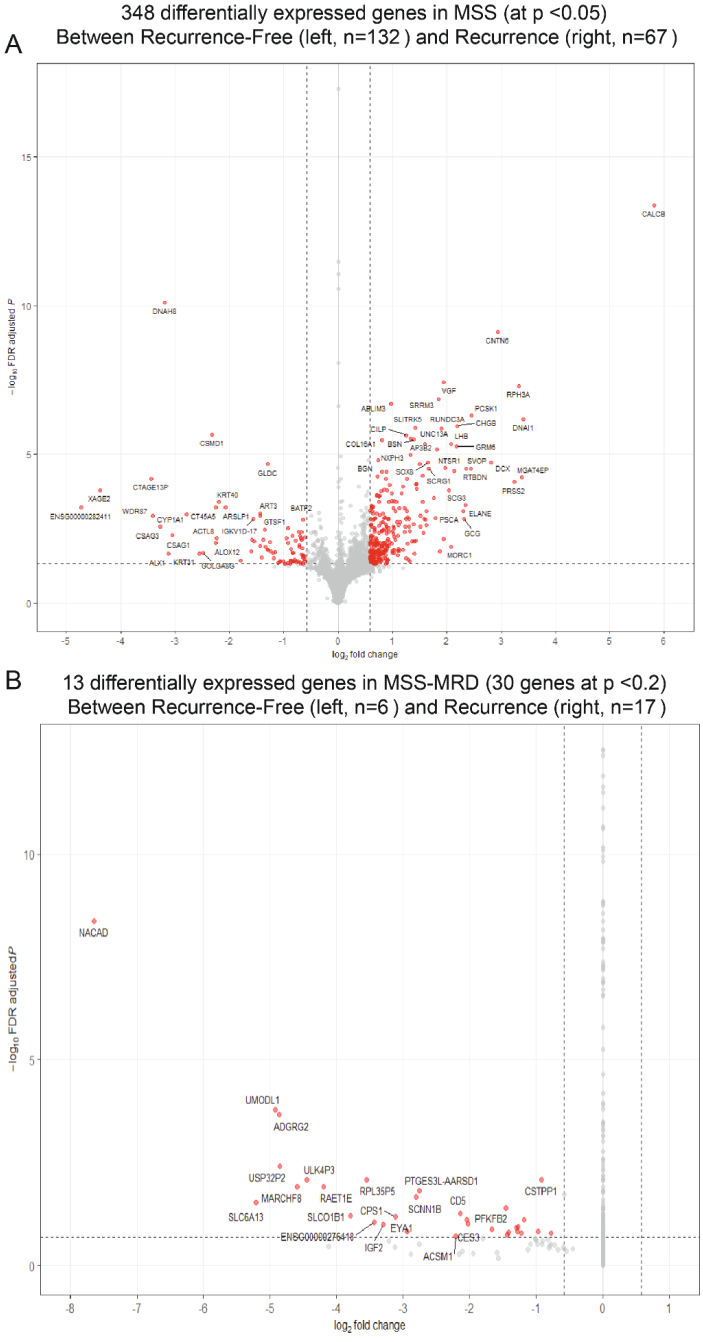
Supplementary Figure 1: Flowchart depicting inclusion criteria from 259 patients with RNA sequencing to the final cohort of 244 patients with sufficient RNA sequencing quality and known MSS/MMR status. This cohort was separated in an MSS and MSI cohort, to analyze prognostic value of biomarkers for recurrence. Based on post-surgery ctDNA detection, a sub-cohort of patients with MRD was defined. Only MSS-MRD contained sufficient patients to analyze predictive value of biomarkers for response to ACT. In each cohort, the availability of transcriptomic and genomic biomarkers is indicated.
IHC, immunohistochemistry; MMR, mismatch repair; MRD, minimal residual disease; MSI, microsatellite instable; MSS, microsatellite stable.



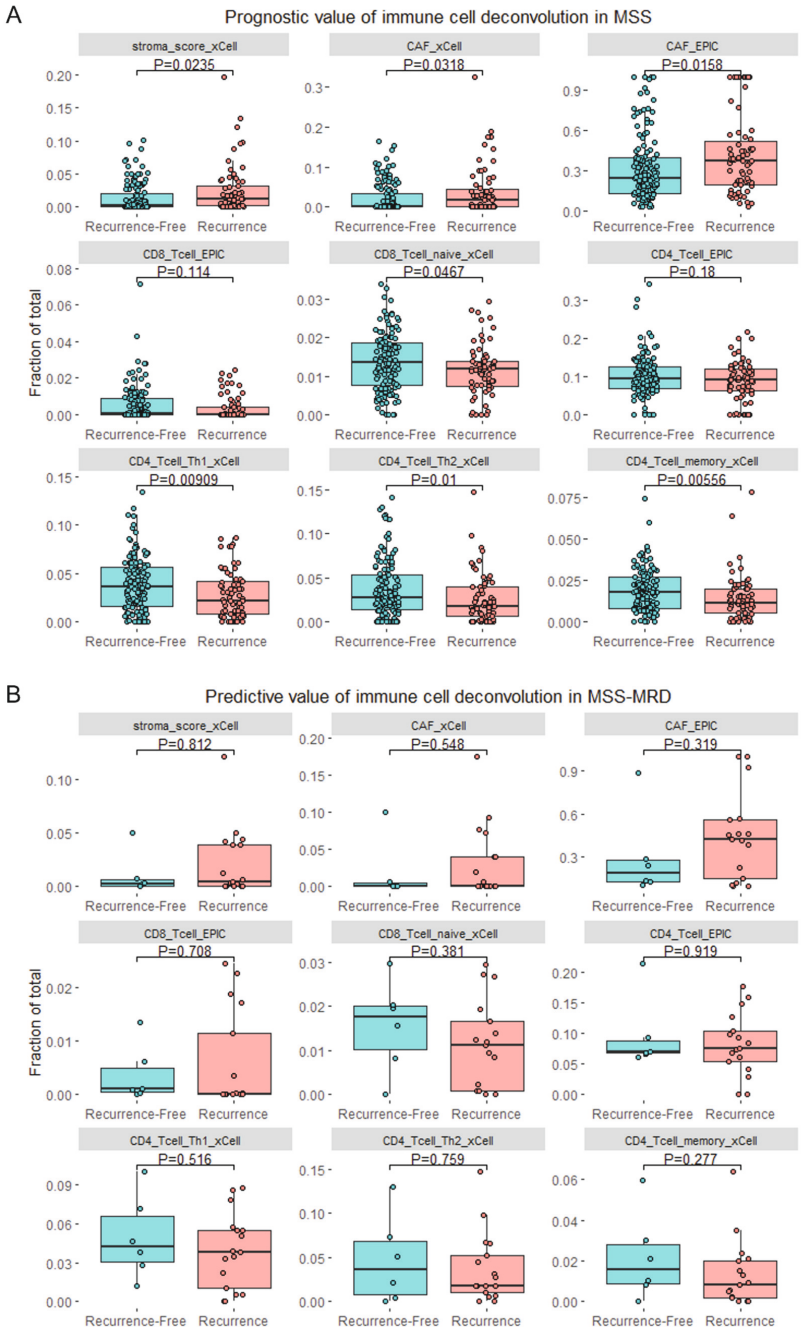
Supplementary Figure 2: Quality control of FFPE RNA sequencing data: A) The mean Pearson correlation of each sample to all other samples in the dataset was calculated and DGE analyses (using CIBRA) for known biologically relevant comparisons were done iteratively while excluding increasing numbers of samples at increasing thresholds of the mean Pearson correlation (left to right). When excluding samples with a mean Pearson correlation >0.65 (dashed red line), the CIBRA impact score (DGE signal) in each comparison starts to increase, while the non-specific DGE signal (based on 30 random permutations) starts to decrease. The 14 samples with a mean Pearson correlation ≤0.65 to other samples were therefore determined not to contribute meaningfully to DGE analyses and were hence excluded; B) The inferior quality of the 14 excluded samples (red lines) was confirmed based on low RNA library complexity (left) and gene coverage (right) compared to the other samples (black lines).
DGE, differential gene expression; MUT, mutant; WT, wild type.



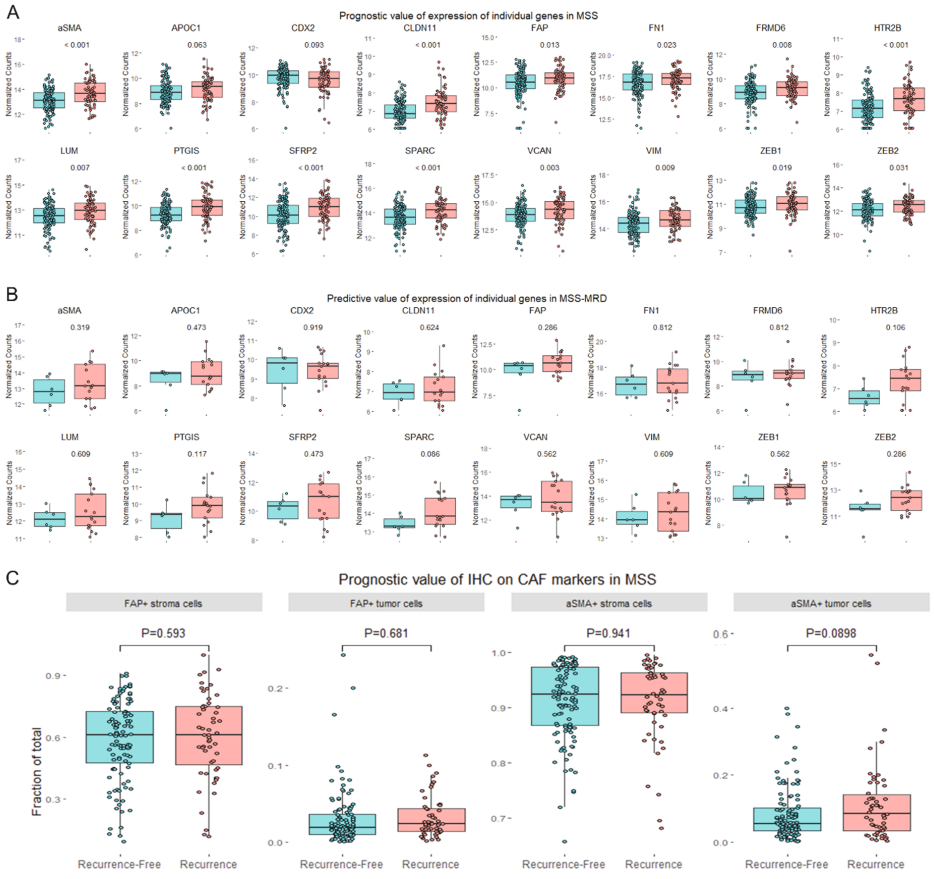
Supplementary Figure 3: Volcano plot depicting DEGs based on DESeq2, genes with positive differential expression in patients with recurrence are plotted to the right, in A) MSS and B) MSS-MRD. *DEGs, differentially expressed genes; FDR, false discovery rate; MSS, microsatellite stable.*



Supplementary Figure 4: Immune cell deconvolution based on EPIC and xCell: significant differences in patients with versus without recurrence (based on Mann-Whitney U test) or plotted in A) MSS and B) MSS-MRD.
CAF, cancer-associated fibroblast; CD, cluster of differentiation; Th, T helper cell.



Supplementary Figure 5: Individual fibroblast markers per recurrence status (Mann-Whitney U): Gene expression of individual mesenchymal genes involved in the CAF signature (APOC1, CLDN11, PTGIS, SFRP2, LUM) and/or EMT (CDX2, FN1, LUM, VIM, ZEB1, ZEB2), known to be expressed only in neoplastic cells (HTR2B, FMRD6) or also stromal cells (LUM, SPARC, VCAN), including fibroblast markers (αSMA and FAP) in A) MSS and B) MSS-MRD; C) IHC protein expression of αSMA en FAP on tissue microarrays of resected MSS tumors.
αSMA, α smooth muscle actin; APOC1, apolipoprotein C1; CDX2, caudal type homeobox 2; CLDN11, claudin 11; FAP, fibroblast activation protein; FRMD6, FERM domain containing 6; FN1, fibronectin 1; HTR2B, 5-hydroxytryptamine receptor 2B; IHC, immunohistology; LUM, lumican; MRD, minimal residual disease; MSS, microsatellite stable; PTGIS, prostaglandin I2 synthase; SFRP2, secreted frizzled-related protein 2; SPARC, secreted protein acidic and cysteine rich; VCAN, versican; VIM, vimentin; ZEB1/2, zinc finger E-box binding homeobox 1/2.



Supplementary Table 1: Patient and tumor characteristics per cohort based on MMR/MSS status and ctDNA-based MRD. *MRD, minimal residual disease; MSI, microsatellite instable; MSS, microsatellite stable; SD, standard deviation.*

	MSS (n=199)	MSS-MRD (n=23)	MSI (n=45)
Age mean (SD)	62.7 (9.7)	65.3 (9, 9)	64.4 (8.8)
Sex			
Female	79 (40%)	7 (30%)	28 (67%)
Male	120 (60%)	16 (70%)	15 (33%)
Clinical risk			
T1-3N1	119 (70%)	12 (52%)	28 (62%)
T4/N2	80 (40%)	11 (48%)	17 (38%)
T			
T1	5 (2,5%)	0 (0%)	0 (0%)
T2	32 (16%)	3 (13%)	0 (0%)
T3	115 (58%)	13 (57%)	33 (73%)
T4	47 (24%)	7 (30%)	12 (27%)
N			
N1	142 (71%)	14 (61%)	38 (84%)
N2	57 (29%)	9 (39%)	7 (16%)
Side			
left	126 (63%)	11 (48%)	10 (22%)
right	73 (37%)	12 (52%)	35 (78%)
Histology			
Adenocarcinoma	178 (89%)	21 (91%)	28 (62%)
Other	21 (11%)	2 (8,7%)	17 (38%)
Differentiation			
Moderate	178 (91%)	20 (87%)	25 (60%)
Poor	18 (9%)	3 (13%)	18 (40%)
Missing	3	0	2
Lymphatic or angioinvasion			
No	92 (46%)	10 (43%)	18 (40%)
Yes	98 (54%)	13 (57%)	26 (60%)
Missing	9	0	1
Radical Resection			
R0	190 (98%)	22 (100%)	45 (100%)
R1 or R2	4 (2%)	0 (0%)	0 (0%)
Missing	5	1	0
Chemo			
CAPOX	180 (91%)	17 (73%)	45 (100%)
Capecitabine	19 (9)	6 (27%)	0 (0%)
Recurrence			
Local or regional	5 (3%)	0 (0%)	2 (4%)
Metastatic	62 (31%)	17 (100%)	4 (8%)
Liver	20 (32%)	10 (59%)	0 (0%)
Lung	14 (23%)	1 (6%)	1 (25%)
Peritoneum	12 (19%)	3 (18%)	1 (25%)
Lymph	2 (3%)	1 (6%)	0 (0%)
Multiple	8 (12%)	2 (12%)	0 (0%)
Other	6 (9%)	0 (0%)	2 (50%)

Supplementary Table 2: Results from differential gene expression analysis between patients with recurrence (positive fold change) and without recurrence in MSS cohort. Significant results are shown in Supplementary Figure 3, other genes will be available online with the publication of the manuscript and are deposited in EGAS50000000804.

Supplementary Table 3: Results from differential gene expression analysis between patients with recurrence (positive fold change) and without recurrence in MSS-MRD. Significant results are shown in Supplementary Figure 3, other genes will be available online with the publication of the manuscript and are deposited in EGAS50000000804.

Supplementary Table 4: Results from gene set variation analysis (GSVA), showing GSVA score difference of all 50MSigDB hallmark gene sets. Per cohort based on MMR/MSS status and ctDNA-based MRD. *FDR, false discovery rate; p, p-value.*

	MSS			MSS-MRD			MSI		
	GSVA	p	FDR	GSVA	p	FDR	GSVA	p	FDR
ADIPOGENESIS	-0.031	0.150	0.227	-0.059	0.370	0.551	0.053	0.632	0.954
ALLOGRAFT_REJECTION	-0.014	0.407	0.484	0.003	0.957	0.977	-0.050	0.497	0.954
ANDROGEN_RESPONSE	0.025	0.307	0.384	-0.002	0.982	0.982	0.081	0.389	0.954
ANGIOGENESIS	0.163	0.000	0.001	0.300	0.028	0.176	0.073	0.641	0.954
APICAL_JUNCTION	0.099	0.000	0.000	0.133	0.131	0.327	0.088	0.340	0.954
APICAL_SURFACE	0.008	0.731	0.777	-0.016	0.826	0.918	0.057	0.516	0.954
APOPTOSIS	0.040	0.046	0.075	0.011	0.881	0.928	0.041	0.654	0.954
BILE_ACID_METABOLISM	-0.042	0.031	0.052	-0.167	0.030	0.176	0.076	0.385	0.954
CHOLESTEROL_HOMEOSTASIS	-0.011	0.727	0.777	-0.187	0.037	0.176	0.027	0.827	0.954
COAGULATION	0.066	0.008	0.015	0.133	0.120	0.316	0.042	0.682	0.954
COMPLEMENT	0.027	0.272	0.360	0.107	0.168	0.381	-0.021	0.827	0.954
DNA_REPAIR	-0.087	0.000	0.001	-0.131	0.082	0.275	-0.107	0.279	0.954
E2F_TARGETS	-0.256	0.000	0.000	-0.301	0.044	0.182	-0.174	0.293	0.954
EPITHELIAL_MESENCHYMAL_TRANSITION	0.178	0.001	0.002	0.225	0.175	0.381	0.203	0.256	0.954
ESTROGEN_RESPONSE_EARLY	0.044	0.010	0.019	-0.007	0.891	0.928	0.021	0.806	0.954
ESTROGEN_RESPONSE_LATE	-0.014	0.420	0.488	-0.060	0.232	0.430	-0.073	0.384	0.954
FATTY_ACID_METABOLISM	-0.102	0.000	0.000	-0.198	0.014	0.176	0.017	0.879	0.954
G2M_CHECKPOINT	-0.218	0.000	0.000	-0.233	0.111	0.316	-0.142	0.365	0.954
GLYCOLYSIS	-0.024	0.267	0.360	-0.058	0.386	0.551	-0.011	0.911	0.954
HEDGEHOG_SIGNALING	0.146	0.000	0.000	0.151	0.259	0.430	0.024	0.844	0.954
HEME_METABOLISM	0.052	0.002	0.005	0.122	0.030	0.176	-0.008	0.916	0.954
HYPOXIA	0.086	0.004	0.008	0.080	0.381	0.551	0.033	0.755	0.954
IL2_STAT5_SIGNALING	0.015	0.550	0.611	0.057	0.482	0.635	-0.046	0.607	0.954
IL6_JAK_STAT3_SIGNALING	-0.001	0.964	0.964	0.107	0.253	0.430	-0.056	0.620	0.954
INFLAMMATORY_RESPONSE	0.004	0.911	0.949	0.113	0.230	0.430	-0.073	0.537	0.954
INTERFERON_ALPHA_RESP	-0.038	0.220	0.323	-0.043	0.536	0.687	-0.057	0.658	0.954
INTERFERON_GAMMA_RESP	-0.029	0.273	0.360	0.033	0.631	0.733	-0.042	0.707	0.954
KRAS_SIGNALING_DN	0.015	0.327	0.399	0.083	0.119	0.316	0.011	0.879	0.954
KRAS_SIGNALING_UP	0.066	0.007	0.014	0.060	0.477	0.635	0.003	0.973	0.993
MITOTIC_SPINDLE	-0.022	0.229	0.328	-0.043	0.470	0.635	0.012	0.903	0.954
MTORC1_SIGNALING	-0.112	0.001	0.002	-0.288	0.003	0.082	-0.109	0.410	0.954
MYC_TARGETS_V1	-0.190	0.000	0.000	-0.261	0.050	0.192	-0.152	0.292	0.954
MYC_TARGETS_V2	-0.239	0.000	0.000	-0.325	0.037	0.176	-0.249	0.216	0.954
MYOGENESIS	0.134	0.000	0.000	0.227	0.021	0.176	0.087	0.434	0.954
NOTCH_SIGNALING	0.088	0.001	0.002	0.042	0.587	0.698	0.119	0.206	0.954
OXIDATIVE_PHOSPHORYLATION	-0.143	0.000	0.001	-0.208	0.116	0.316	-0.055	0.705	0.954

	MSS			MSS-MRD			MSI		
	GSVA	p	FDR	GSVA	p	FDR	GSVA	p	FDR
P53_PATHWAY	0.022	0.286	0.366	-0.078	0.267	0.430	0.029	0.764	0.954
PANCREAS_BETA_CELLS	-0.002	0.943	0.963	0.098	0.236	0.430	-0.084	0.371	0.954
PEROXISOME	-0.085	0.000	0.001	-0.238	0.003	0.082	0.069	0.447	0.954
PI3K_AKT_MTOR_SIGNALING	-0.058	0.001	0.003	-0.137	0.033	0.176	-0.038	0.641	0.954
PROTEIN_SECRETION	0.018	0.525	0.597	-0.012	0.889	0.928	-0.051	0.653	0.954
REACTIVE_OXYGEN_SPECIES	-0.054	0.019	0.033	-0.099	0.189	0.393	-0.034	0.751	0.954
SPERMATOGENESIS	-0.061	0.001	0.002	-0.061	0.355	0.551	-0.060	0.433	0.954
TGF_BETA_SIGNALING	0.120	0.000	0.000	0.112	0.265	0.430	0.055	0.661	0.954
TNFA_SIGNALING_VIA_NFKB	0.071	0.030	0.052	0.046	0.647	0.735	-0.050	0.682	0.954
UNFOLDED_PROTEIN_RESP	-0.098	0.000	0.001	-0.147	0.156	0.372	-0.166	0.191	0.954
UV_RESPONSE_DN	0.134	0.000	0.000	0.200	0.039	0.176	0.106	0.281	0.954
UV_RESPONSE_UP	-0.053	0.003	0.006	-0.083	0.073	0.260	-0.038	0.631	0.954
WNT_BETA_CATENIN_SIGN	0.041	0.117	0.183	-0.062	0.576	0.698	0.000	0.997	0.997
XENOBIOTIC_METABOLISM	-0.044	0.010	0.019	-0.031	0.585	0.698	0.055	0.511	0.954

Supplementary Table 5: Results of all immune cell deconvolutions, per cohort based on MMR/MSS status and ctDNA-based MRD, comparing patients with versus without recurrence.
FC, FoldChange; p, p-value; R, Wilcox effect size.

	MSS			MSS-MRD			MSI		
cell_type	FC	R	p	FC	R	p	FC	R	p
EPIC									
Bcells	1.293	0.015	0.832	0.979	0.044	0.865	0.124	0.423	0.565
Cancer-associated fibroblasts	1.533	0.171	0.016	2.258	0.219	0.319	0.314	0.034	9.105
CD4_Tcells	0.957	0.095	0.180	1.078	0.029	0.919	0.045	0.782	0.057
CD8_Tcells	0.499	0.112	0.114	0.024	0.088	0.708	0.119	0.442	0.270
Endothelial	1.084	0.017	0.810	0.975	0.029	0.919	0.010	0.961	0.832
Macrophages	0.943	0.002	0.974	1.128	0.102	0.658	0.209	0.170	0.636
Natural killer cells	1.355	0.054	0.449	4.428	0.263	0.227	0.085	0.591	0.143
otherCells	0.813	0.168	0.018	0.648	0.190	0.392	0.333	0.024	0.439
xCELL									
B cell	1.461	0.001	0.993	0.010	0.219	0.310	0.219	0.310	0.005
B cell memory	0.313	0.041	0.565	0.038	0.045	0.858	0.045	0.858	0.063
B cell naive	0.033	0.056	0.431	0.116	0.263	0.221	0.263	0.221	0.000
B cell plasma	0.765	0.228	0.001	0.972	0.044	0.865	0.044	0.865	0.427
Cancer associated fibroblast	1.819	0.152	0.032	1.735	0.117	0.549	0.117	0.098	1.201
Class-switched memory B cell	1.475	0.074	0.296	1.024	0.000	1.000	0.000	1.000	0.626
Common lymphoid progenitor	0.827	0.186	0.009	0.705	0.168	0.441	0.168	0.441	0.690
Common myeloid progenitor	0.000	0.053	0.454	0.798	0.281	0.189	0.281	0.189	1.488
Endothelial cell	1.231	0.022	0.757	1.987	0.037	0.888	0.037	0.888	0.041
Eosinophil	3.429	0.091	0.201	0.075	0.113	0.615	0.113	0.615	0.183
Granulocyte-monocyte progenitor	1.045	0.044	0.533	0.000	0.406	0.056	0.406	0.056	0.345
Hematopoietic stem cell	1.142	0.119	0.093	1.037	0.117	0.609	0.117	0.609	0.192
Immune score	0.967	0.012	0.865	1.151	0.058	0.812	0.058	0.812	0.679
Macrophage	1.080	0.013	0.852	2.294	0.133	0.546	0.133	0.546	0.244
Macrophage M1	1.150	0.024	0.732	1.460	0.118	0.594	0.118	0.594	0.447
Macrophage M2	1.330	0.171	0.016	0.464	0.102	0.658	0.102	0.658	0.852
Mast cell	1.022	0.066	0.356	1.437	0.365	0.087	0.365	0.087	0.031
Microenvironment score	1.155	0.107	0.131	1.864	0.146	0.516	0.146	0.516	0.283
Monocyte	2.836	0.092	0.196	11.046	0.077	0.739	0.077	0.739	0.105
Myeloid dendritic cell	0.009	0.070	0.321	0.528	0.067	0.775	0.067	0.775	0.087
Myeloid dendritic cell activated	1.016	0.046	0.517	1.264	0.044	0.861	0.044	0.861	0.051
Neutrophil	1.125	0.041	0.564	0.000	0.050	0.842	0.050	0.842	0.059
Natural Killer cell	0.038	0.002	0.977	0.203	0.030	0.914	0.030	0.914	0.000

	MSS			MSS-MRD			MSI		
cell_type	**FC**	**R**	**p**	**FC**	**R**	**p**	**FC**	**R**	**p**
Plasmacytoid dendritic cell	0.834	0.010	0.892	0.032	0.062	0.795	0.062	0.795	0.078
stroma score	4.538	0.161	0.024	1.652	0.015	0.813	0.015	0.973	0.015
T cell CD4+ (non-regulatory)	0.000	0.060	0.395	0.000	0.007	1.000	0.007	1.000	0.007
T cell CD4+ central memory	1.830	0.077	0.281	0.572	0.147	0.504	0.147	0.504	0.291
T cell CD4+ effector memory	0.140	0.150	0.034	2.692	0.044	0.859	0.044	0.859	0.287
T cell CD4+ memory	0.656	0.197	0.006	0.518	0.198	0.278	0.198	0.005	0.000
T cell CD4+ naive	0.000	0.006	0.937	0.587	0.211	0.330	0.211	0.330	0.274
T cell CD4+ Thelper 1	0.615	0.185	0.009	0.896	0.146	0.516	0.146	0.516	0.021
T cell CD4+ Thelper 2	0.643	0.183	0.010	0.504	0.132	0.760	0.132	0.550	0.221
T cell CD8+	0.066	0.052	0.467	5.384	0.355	0.095	0.355	0.095	0.403
T cell CD8+ central memory	0.780	0.024	0.735	0.399	0.241	0.263	0.241	0.263	0.915
T cell CD8+ effector memory	0.033	0.066	0.354	0.638	0.105	0.639	0.105	0.639	0.869
T cell CD8+ naive	0.873	0.141	0.047	17.564	0.161	0.382	0.161	0.473	0.566
T cell gamma delta	0.508	0.104	0.142	0.032	0.117	0.598	0.117	0.598	0.196
T cell NK	0.767	0.072	0.309	0.564	0.029	0.916	0.029	0.916	0.032
T cell regulatory (Tregs)	0.913	0.052	0.460	0.112	0.075	0.748	0.075	0.748	0.628

Supplementary Table 6: Histological results including the fraction of FAP+ and aSMA+ cells on for IHC on TMAs (median and IQR), per cohort based on MMR/MSS status and ctDNA-based MRD.
αSMA, α smooth muscle actin; FAP, fibroblast activation protein; MRD, minimal residual disease; MSI, microsatellite instable; MSS, microsatellite stable.

	MSS			MSS-MRD			MSI		
	Free (n=132)	**Recurrence (n=67)**	**P-value**	**Free (n=6)**	**Recurrence (n=17)**	**P-value**	**Free (n=39)**	**Recurrence (n=6)**	**P-value**
Stroma cells FAP+	0.613 [0.475-0.725]	0.612 [0.465-0.750]	0.593	0.667 [0.541-0.712]	0.533 [0.387-0.705]	0.330	0.702 [0.552-0.795]	0.586 [0.584-0.724]	0.692
Neoplastic cells FAP+	0.018 [0.009-0.036]	0.023 [0.014-0.043]	0.681	0.021 [0.012-0.028]	0.022 [0.009-0.041]	0.726	0.038 [0.021-0.088]	0.033 [0.029-0.038]	1.000
Stroma cells αSMA+	0.924 [0.867-0.973]	0.923 [0.890-0.963]	0.941	0.939 [0.851-0.968]	0.923 [0.836-0.976]	0.731	0.932 [0.840-0.961]	0.859 [0.829-0.890]	0.134
Neoplastic cells αSMA+	0.055 [0.033-0.101]	0.085 [0.033-0.142]	0.090	0.087 [0.047-0.099]	0.116 [0.054-0.183]	0.303	0.082 [0.029-0.116]	0.134 [0.100-0.151]	0.228

Supplementary Table 7: Single nucleotide variants in cancer-related genes, affecting >5% of patients or providing targets for alternative treatment. Per cohort based on MMR/MSS status and ctDNA-based MRD. *mut, mutant; wt, wildtype.*

	MSS			MSS-MRD			MSI		
	Free (n=132)	**Recurrence (n=67)**	**P-value**	**Free (n=6)**	**Recurrence (n=17)**	**P-value**	**Free (n=39)**	**Recurrence (n=6)**	**P-value**
APC			0.443			1			1
wt	108 (82.4%)	50 (76.9%)		1 (16.7%)	3 (17.6%)		27 (73.0%)	5 (83.3%)	
mut	23 (17.6%)	15 (23.1%)		5 (83.3%)	14 (82.4%)		10 (27.0%)	1 (16.7%)	
TP53			1			0.318			0.403
wt	40 (30.5%)	19 (29.2%)		3 (50.0%)	4 (23.5%)		23 (62.2%)	5 (83.3%)	
mut	91 (69.5%)	46 (70.8%)		3 (50.0%)	13 (76.5%)		14 (37.8%)	1 (16.7%)	
KRAS			0.765			1			1
wt	70 (53.4%)	34 (50.7%)		3 (50.0%)	8 (47.1%)		34 (89.5%)	6 (100%)	
mut	61 (46.6%)	33 (49.3%)		3 (50.0%)	9 (52.9%)		4 (10.5%)	0 (0%)	
PIK3CA			0.33			1			1
wt	105 (80.2%)	56 (86.2%)		5 (83.3%)	13 (76.5%)		27 (73.0%)	5 (83.3%)	
mut	26 (19.8%)	9 (13.8%)		1 (16.7%)	4 (23.5%)		10 (27.0%)	1 (16.7%)	

	MSS Free	Recurrence	P	MSS-MRD Free	Recurrence	P	MSI Free	Recurrence	P
SOX9			0.546			0.621			0.659
wt	108 (82.4%)	56 (86.2%)		5 (83.3%)	11 (64.7%)		26 (70.3%)	5 (83.3%)	
mut	23 (17.6%)	9 (13.8%)		1 (16.7%)	6 (35.3%)		11 (29.7%)	1 (16.7%)	
SMAD4			0.148			0.539			0.262
wt	120 (91.6%)	55 (84.6%)		6 (100%)	14 (82.4%)		36 (97.3%)	5 (83.3%)	
mut	11 (8.4%)	10 (15.4%)		0 (0%)	3 (17.6%)		1 (2.7%)	1 (16.7%)	
FBXW7			0.783			1			1
wt	121 (92.4%)	59 (90.8%)		6 (100%)	15 (88.2%)		21 (56.8%)	4 (66.7%)	
mut	10 (7.6%)	6 (9.2%)		0 (0%)	2 (11.8%)		16 (43.2%)	2 (33.3%)	
NRAS			0.687			1			NA
wt	127 (96.9%)	62 (95.4%)		6 (100%)	15 (88.2%)		37 (100%)	6 (100%)	
mut	4 (3.1%)	3 (4.6%)		0 (0%)	2 (11.8%)		0 (0%)	0 (0%)	
BRAF			0.0192			0.576			1
wt	121 (92.4%)	54 (80.6%)		4 (66.7%)	14 (82.4%)		13 (34.2%)	2 (33.3%)	
mut	10 (7.6%)	13 (19.4%)		2 (33.3%)	3 (17.6%)		25 (65.8%)	4 (66.7%)	
CDKN2A			0.332			NA			1
wt	131 (100%)	64 (98.5%)		6 (100%)	17 (100%)		36 (97.3%)	6 (100%)	
mut	0 (0%)	1 (1.5%)		0 (0%)	0 (0%)		1 (2.7%)	0 (0%)	
CTNNB1			1			NA			NA
wt	130 (99.2%)	64 (98.5%)		6 (100%)	17 (100%)		37 (100%)	6 (100%)	
mut	1 (0.8%)	1 (1.5%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)	
SMAD2			0.4			1			NA
wt	128 (97.7%)	62 (95.4%)		6 (100%)	16 (94.1%)		37 (100%)	6 (100%)	
mut	3 (2.3%)	3 (4.6%)		0 (0%)	1 (5.9%)		0 (0%)	0 (0%)	
ATM			0.754			0.0593			1
wt	122 (93.1%)	62 (95.4%)		4 (66.7%)	17 (100%)		29 (78.4%)	5 (83.3%)	
mut	9 (6.9%)	3 (4.6%)		2 (33.3%)	0 (0%)		8 (21.6%)	1 (16.7%)	
ARID1A			0.687			NA			0.669
wt	127 (96.9%)	62 (95.4%)		6 (100%)	17 (100%)		19 (51.4%)	4 (66.7%)	
mut	4 (3.1%)	3 (4.6%)		0 (0%)	0 (0%)		18 (48.6%)	2 (33.3%)	
MYC			1			NA			NA
wt	122 (93.1%)	61 (93.8%)		6 (100%)	17 (100%)		37 (100%)	6 (100%)	
mut	9 (6.9%)	4 (6.2%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)	
AMER1			0.556			1			0.567
wt	123 (93.9%)	59 (90.8%)		6 (100%)	15 (88.2%)		30 (81.1%)	6 (100%)	
mut	8 (6.1%)	6 (9.2%)		0 (0%)	2 (11.8%)		7 (18.9%)	0 (0%)	
ERBB2			0.483			0.462			1
wt	126 (96.2%)	61 (93.8%)		5 (83.3%)	16 (94.1%)		35 (94.6%)	6 (100%)	
mut	5 (3.8%)	4 (6.2%)		1 (16.7%)	1 (5.9%)		2 (5.4%)	0 (0%)	
PTEN			0.172			0.261			1
wt	126 (96.2%)	65 (100%)		5 (83.3%)	17 (100%)		31 (83.8%)	5 (83.3%)	
mut	5 (3.8%)	0 (0%)		1 (16.7%)	0 (0%)		6 (16.2%)	1 (16.7%)	
CCND2			0.184			1			NA
wt	126 (96.2%)	59 (90.8%)		6 (100%)	15 (88.2%)		37 (100%)	6 (100%)	
mut	5 (3.8%)	6 (9.2%)		0 (0%)	2 (11.8%)		0 (0%)	0 (0%)	
FGFR1			0.666			NA			NA
wt	126 (96.2%)	64 (98.5%)		6 (100%)	17 (100%)		37 (100%)	6 (100%)	
mut	5 (3.8%)	1 (1.5%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)	
FGFR3			1			NA			0.125
wt	131 (99.2%)	67 (100%)		6 (100%)	17 (100%)		36 (92.3%)	4 (66.7%)	
mut	1 (0.8%)	0 (0%)		0 (0%)	0 (0%)		3 (7.7%)	2 (33.3%)	
FGFR4			1			0.261			1
wt	129 (98.5%)	65 (100%)		5 (83.3%)	17 (100%)		35 (94.6%)	6 (100%)	
mut	2 (1.5%)	0 (0%)		1 (16.7%)	0 (0%)		2 (5.4%)	0 (0%)	
SMAD3			0.304			NA			1
wt	127 (96.9%)	65 (100%)		6 (100%)	17 (100%)		36 (97.3%)	6 (100%)	
mut	4 (3.1%)	0 (0%)		0 (0%)	0 (0%)		1 (2.7%)	0 (0%)	
EGFR			0.4			1			NA
wt	128 (97.7%)	62 (95.4%)		6 (100%)	16 (94.1%)		37 (100%)	6 (100%)	
mut	3 (2.3%)	3 (4.6%)		0 (0%)	1 (5.9%)		0 (0%)	0 (0%)	
PDGFRA			1			NA			NA
wt	130 (99.2%)	65 (100%)		6 (100%)	17 (100%)		37 (100%)	6 (100%)	
mut	1 (0.8%)	0 (0%)		0 (0%)	0 (0%)				
PDGFRB			1			NA			NA
wt	130 (99.2%)	65 (100%)		6 (100%)	17 (100%)		37 (100%)	6 (100%)	

mut	1 (0.8%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)	
PIK3R1			1			0.261			0.044
wt	128 (97.7%)	63 (96.9%)		5 (83.3%)	17 (100%)		33 (89.2%)	3 (50.0%)	
mut	3 (2.3%)	2 (3.1%)		1 (16.7%)	0 (0%)		4 (10.8%)	3 (50.0%)	
CCND3			1			NA			1
wt	128 (97.7%)	64 (98.5%)		6 (100%)	17 (100%)		36 (97.3%)	6 (100%)	
mut	3 (2.3%)	1 (1.5%)		0 (0%)	0 (0%)		1 (2.7%)	0 (0%)	
CCND1			0.601			0.261			1
wt	129 (98.5%)	63 (96.9%)		5 (83.3%)	17 (100%)		36 (97.3%)	6 (100%)	
mut	2 (1.5%)	2 (3.1%)		1 (16.7%)	0 (0%)		1 (2.7%)	0 (0%)	
RNF43			0.107			1			0.248
wt	130 (99.2%)	62 (95.4%)		6 (100%)	15 (88.2%)		5 (13.5%)	2 (33.3%)	
mut	1 (0.8%)	3 (4.6%)		0 (0%)	2 (11.8%)		32 (86.5%)	4 (66.7%)	
SMARCA4			0.304			NA			1
wt	127 (96.9%)	65 (100%)		6 (100%)	17 (100%)		32 (86.5%)	5 (83.3%)	
mut	4 (3.1%)	0 (0%)		0 (0%)	0 (0%)		5 (13.5%)	1 (16.7%)	
MSH3			1			NA			0.412
wt	130 (99.2%)	65 (100%)		6 (100%)	17 (100%)		20 (54.1%)	2 (33.3%)	
mut	1 (0.8%)	0 (0%)		0 (0%)	0 (0%)		17 (45.9%)	4 (66.7%)	
KMT2D			1			NA			1
wt	130 (99.2%)	65 (100%)		6 (100%)	17 (100%)		21 (56.8%)	3 (50.0%)	
mut	1 (0.8%)	0 (0%)		0 (0%)	0 (0%)		16 (43.2%)	3 (50.0%)	
PRKDC			1			NA			0.164
wt	129 (98.5%)	64 (98.5%)		6 (100%)	17 (100%)		29 (78.4%)	3 (50.0%)	
mut	2 (1.5%)	1 (1.5%)		0 (0%)	0 (0%)		8 (21.6%)	3 (50.0%)	
BCORL			1			NA			1
wt	130 (99.2%)	65 (100%)		6 (100%)	17 (100%)		24 (64.9%)	4 (66.7%)	
mut	1 (0.8%)	0 (0%)		0 (0%)	0 (0%)		13 (35.1%)	2 (33.3%)	
SPEN			NA			NA			1
wt	131 (100%)	65 (100%)		6 (100%)	17 (100%)		26 (70.3%)	4 (66.7%)	
mut	0 (0%)	0 (0%)		0 (0%)	0 (0%)		11 (29.7%)	2 (33.3%)	
TGFBR1			1			NA			
wt	130 (99.2%)	65 (100%)		6 (100%)	17 (100%)				NA
mut	1 (0.8%)	0 (0%)		0 (0%)	0 (0%)		37 (100%)	6 (100%)	
TGFBR2			1			NA			0.393
wt	130 (99.2%)	64 (98.5%)		6 (100%)	17 (100%)		16 (43.2%)	4 (66.7%)	
mut	1 (0.8%)	1 (1.5%)		0 (0%)	0 (0%)		21 (56.8%)	2 (33.3%)	
B2M			1			1			0.649
wt	130 (99.2%)	64 (98.5%)		6 (100%)	16 (94.1%)		25 (67.6%)	5 (83.3%)	
mut	1 (0.8%)	1 (1.5%)		0 (0%)	1 (5.9%)		12 (32.4%)	1 (16.7%)	
BRCA2			1			1			1
wt	128 (97.7%)	63 (96.9%)		6 (100%)	16 (94.1%)		29 (78.4%)	5 (83.3%)	
mut	3 (2.3%)	2 (3.1%)		0 (0%)	1 (5.9%)		8 (21.6%)	1 (16.7%)	
BRCA1			NA			NA			1
wt	131 (100%)	65 (100%)		6 (100%)	17 (100%)		35 (94.6%)	6 (100%)	
mut	0 (0%)	0 (0%)		0 (0%)	0 (0%)		2 (5.4%)	0 (0%)	
CASP8			0.332			1			0.659
wt	131 (100%)	64 (98.5%)		6 (100%)	16 (94.1%)		26 (70.3%)	5 (83.3%)	
mut	0 (0%)	1 (1.5%)		0 (0%)	1 (5.9%)		11 (29.7%)	1 (16.7%)	
ASXL1			1			NA			0.309
wt	130 (99.2%)	65 (100%)		6 (100%)	17 (100%)		27 (73.0%)	6 (100%)	
mut	1 (0.8%)	0 (0%)		0 (0%)	0 (0%)		10 (27.0%)	0 (0%)	
ARID5B			0.332			1			0.046
wt	131 (100%)	64 (98,5%)		6 (100%)	16 (94,1%)		36 (97,3%)	4 (66,7%)	
mut	0 (0%)	1 (1,5%)		0 (0%)	1 (5,9%)		1 (2,7%)	2 (33,3%)	

Supplementary Table 8: Patient-level genomic alterations based on targeted DNA sequencing of 505 cancer-related genes for all patients. Combined for MSS and MSS-MRD and MSI cohort. Results will be available online with the publication of the manuscript and are deposited in EGAS50000000804.

Supplementary Table 9: Patient and tumor characteristics per recurrence status in the MSI cohort. *SD, standard deviation.*

	Recurrence-Free (n=39)	Recurrence (n=6)	Fisher's exact
Age			0.880
Mean (SD)	64.1 (9.4)	66.3 (4.6)	
Sex			1
Female	26 (67%)	4 (67%)	
Male	13 (33%)	2 (33%)	
Clinical risk			0.023
T1-3N1	27 (69%)	1 (17%)	
T4/N2	12 (31%)	5 (83%)	
T			0.650
T1	0 (0%)	0 (0%)	
T2	0 (0%)	0 (0%)	
T3	29 (74%)	4 (67%)	
T4	10 (26%)	2 (33%)	
N			0.003
N1	36 (92%)	2 (33%)	
N2	3 (7.7%)	4 (67%)	
Side			1
left	9 (23%)	1 (17%)	
right	30 (77%)	5 (83%)	
Histology			0.658
Adenocarcinoma	25 (64%)	3 (50%)	
Other	14 (36%)	3 (50%)	
Differentiation			0.683
Moderate	22 (59%)	3 (50%)	
Poor	15 (41%)	3 (50%)	
Missing	2	0	
Lymphatic or angioinvasion			1
No	2 (33%)	16 (42%)	
Yes	4 (67%)	22 (58%)	
Missing	0 (0%)	1	
Radical Resection			NA
R0	39 (100%)	6 (100%)	
R1 or R2	0 (0%)	0 (0%)	
Missing	0	0	
Chemo			NA
CAPOX	39 (100%)	6 (100%)	
Capecitabine	0 (0%)	0 (0%)	

Supplementary Table 10: Results from differential gene expression analysis between patients with recurrence (positive fold change) and without recurrence in MSI cohort. Significant results are shown in Main Figure 5, other genes will be available online with the publication of the manuscript and are deposited in EGAS50000000804.

7

Patient-derived colon cancer organoids to model adjuvant chemoresistance and identify predictive biomarkers

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Under review

Abstract

Introduction

The standard of care for patients with high-risk stage II and stage III colon cancer is resection, followed by adjuvant chemotherapy with a fluoropyrimidine (Fp) and oxaliplatin (Ox). However, one-third of patients are still experiencing recurrence due to resistance to FpOx. We investigate the potential of patient-derived organoids (PDOs) to model chemoresistance and to identify biomarkers predicting resistance.

Methods

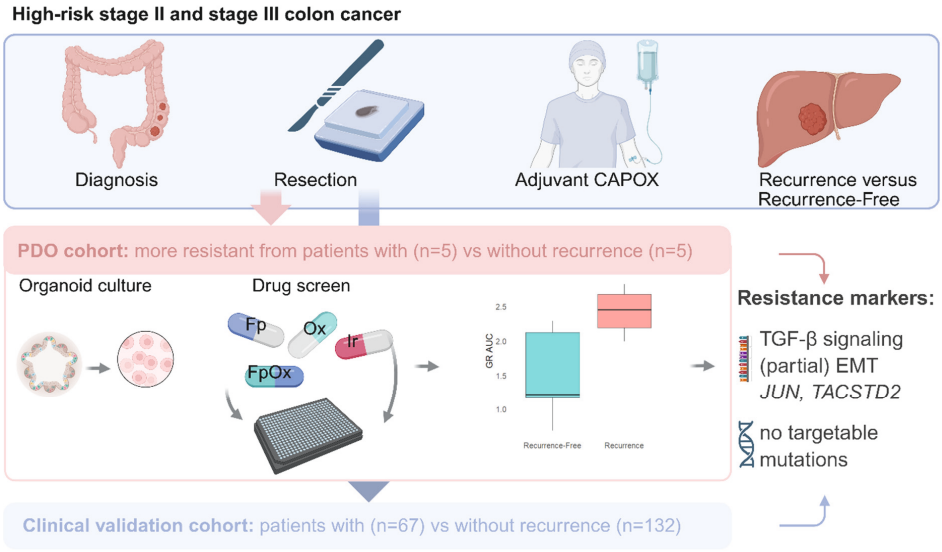
PDOs were established from resection material of treatment-naïve stage II-III colon cancer patients, before they received adjuvant FpOx. PDO drug sensitivity, quantified as area under the growth rate inhibition curve (GR_{AUC}), was compared between patients with (n=5) versus without (n=5) recurrence of disease. PDOs were classified as resistant versus sensitive to FpOx, for comparison of molecular profiles by bulk mRNA sequencing and whole genome sequencing (WGS). Resulting biomarkers were validated in resected colon tumor tissue of a clinical cohort of patients with (n=67) versus without (n=132) recurrence.

Results

PDOs derived from patients with recurrence showed increased resistance to FpOx compared to those without recurrence (GR_{AUC} 2.42 versus 1.50, p=0.035), and in most cases concurrent resistance to irinotecan. Compared to FpOx-sensitive PDOs (n=3), resistant PDOs (n=7) showed higher expression of mesenchymal signatures and genes (i.a. EMT, *JUN*), as well as potentially actionable genes (*TACSTD2* encoding TROP2 – a target for antibody-drug conjugates). WGS revealed no mutations predicting FpOx resistance or enabling alternative targeted treatment.

Conclusion

PDOs derived from colon cancer patients with recurrence of disease after adjuvant FpOx capture tumor-intrinsic resistance to FpOx. This resistance is associated with mesenchymal gene expression, as was validated in a clinical cohort. These findings suggest that PDOs offer a unique platform to study chemoresistance and to explore avenues for potential alternative therapies.


High-risk stage II and stage III colon cancer
Diagnosis
Resection
Adjuvant CAPOX
Recurrence versus Recurrence-Free
PDO cohort: more resistant from patients with (n=5) vs without recurrence (n=5)
Organoid culture
Drug screen
Fp
Ox
Ir
FpOx
GR AUC
2.5
2.0
1.5
1.0
Recurrence-Free
Recurrence
Resistance markers:
TGF-β signaling
(partial) EMT
JUN, TACSTD2
no targetable
mutations
Clinical validation cohort: patients with (n=67) vs without recurrence (n=132)

Introduction

The current standard treatment for high-risk stage II (T4) and stage III (N+) colon cancer is resection followed by adjuvant chemotherapy (ACT). ACT consists of fluoropyrimidine (Fp; capecitabine or 5-fluouracil (5-FU)) as monotherapy[1] or combined with oxaliplatin (Ox)[2]. Half of the patients are cured by surgery alone, whereas one-third of the patients still experience recurrence of disease despite ACT.[3] Recent advances in circulating tumor DNA (ctDNA) allow identification of patients not cured by surgery. These patients have minimal residual disease (MRD) after surgery and depend on sensitivity to FpOx to be cured. Among patients with ctDNA-based MRD, two-thirds do not respond to adjuvant FpOx and experience recurrence.[4-6] Novel biomarkers and models are needed to predict this chemoresistance upfront and to inform effective alternative treatment strategies.

To address this clinical need, various clinical studies have investigated biomarkers for recurrence, but it remains challenging to ascertain predictive value for adjuvant response. Poor prognostic factors include pT4/pN2 stage, right-sidedness[7], lymphatic[8] or vascular[9] invasion, proficient mismatch repair (pMMR)[10], consensus molecular subtype (CMS) 4[11], epithelial mesenchymal transition (EMT)[12], and cancer-associated fibroblasts (CAFs)[13]. Such mesenchymal biomarkers are also associated with recurrence in patients with ctDNA-based MRD after resection, indicating lack of response to adjuvant FpOx.[14,15] Although these clinical studies underline the importance of the tumor microenvironment (TME), the tumor cell-intrinsic determinants of FpOx resistance are difficult to uncover. This highlights the need for *in vitro* models.

Patient-derived organoids (PDOs) provide such a model, recapitulating the three-dimensional architecture and heterogenous genetic and transcriptomic composition of the original tumor.[16,17] PDOs are amenable to high-throughput drug screens, which have been shown to correlate with clinical response in metastatic colorectal cancer (CRC).[18-24] Hence, PDOs can model FpOx response *in vitro*, to study biomarkers that govern response and to identify targets for alternative treatments in resistant CRC.
This study selected PDOs from patients with high-risk stage II and stage III colon cancer with and without recurrence after resection and adjuvant FpOx. The aim was to determine whether PDOs can capture FpOx resistance and help identify predictive biomarkers, which were validated in resection tissue of a parallel clinical cohort.[13]

Methods

Patient derived organoids and clinical data

PDOs from patients who underwent radical resection of stage II-III colon cancer followed by adjuvant FpOx (n=8) or Fp monotherapy (n=2) in 2015-2021 were requested from the HUB-Cancer Biobank (TCBIO 12-093) of the foundation Hubrecht Organoid Biobank. The patients provided written informed consent for the biobanking protocol HUB-Cancer and the release was approved by the TC Bio of the UMC Utrecht (22-638). Clinical data including patient and tumor characteristics were collected from the electronic patient database, by data managers who were blinded to the PDO data. Clinical response was determined in standard clinical follow-up, to compare patients who experienced distant or local recurrence of disease (n=5) to patients who were at least 3 years recurrence-free (n=5). The PDOs were established and passaged as previously described.[17,23] The culturing medium (composition in Supplementary Table 1) was refreshed twice per week. PDO identity was confirmed on an array targeting 64 single-nucleotide polymorphisms (SNPs), using TagMan OpenArray technology in combination with the QuantStudio 12K Flex Real-Time PCR System, at the Utrecht Sequencing Facility (USEQ). The genetic distance between PDO DNA and blood DNA was <5 for all samples, with values assigned as follows: 1 for XX versus XY and YY versus XY, 2 for XX versus YY, −2 for no call, ±0.333 for invalid calls.

Drug screening of patient-derived organoids

Drug screens were initiated four days after passage, harvesting PDOs using 1 mg/mL Dispase (Life Technologies Europe B.V., Zuid-Holland, the Netherlands, #17105041). The harvested PDOs were washed twice with adDMEM+++ and filtered through a 100 µm mesh filter. The filtered PDOs were resuspended in screening medium (composition in Supplementary Table 1) with 10% BME for a final concentration of 10 PDOs/µL in 40 µL of PDO suspension per well. Four PDO lines were dispensed per clear-bottomed black-walled ultra-low-attachment 384-well plate (Corning, Zuid-Holland, the Netherlands, #4588), using an automated Multidrop™ Combi Reagent Dispenser. The drugs, the negative controls (solvent 1% dimethyl sulfoxide or 0.3% Tween) and the positive control (Navitoclax 20 µM) were dispensed using a Tecan D300E dispenser. The drugs were added in technical triplicates of an 8-point logarithmic range of clinically relevant concentrations: 5-FU (Accord, infusion concentrate, dissolved to a concentration of 100mM with PBS to a final concentration

containing 0.3% Tween), oxaliplatin (Fresenius Kabi, infusion concentrate, dissolved to a concentration of 11.3 mM with PBS to a final concentration containing 0.3% Tween), the combination of 5-FU and oxaliplatin (as fixed ratio 1.8:1) and SN-38 (active metabolite of Irinotecan, Selleck Chemicals, S4908) (Supplementary Table 2). On day 0 and day 5, X CellTiter-Glo 3D (Promega, #G9681, 40 µL/ well) was added to subsequently quantify cell viability on a Tecan Spark plate reader with Spectramax.

Only drug screens of sufficient quality, as quantified by a Z'-factor >0.3, were included in the analysis.[25] Growth rate inhibition (GR) metrics were employed to account for differences in proliferation rates between PDO lines and to classify drug effect as cytostatic (GR 0), cytotoxic (GR 0 to -1) or inhibitory (GR 0 to 1).[26] Dose-response curves (DRC) were modeled using *Nplr* (v0.1-8), by calculating the technical triplicate's mean growth rate per concentration. The GR_{AUC} (area under the nonfitted 'curve' of the raw GR values) was calculated and normalized using the maximum and minimum GR_{AUC} per drug. Heatmaps illustrating the normalized GR_{AUC} per drug and per PDO were created with *Pheatmap (*v1.0.12) and *ComplexHeatmap* (v3.19). Per drug, the GR_{AUC} was compared between PDOs derived from patients with versus without recurrence using two-sided independent t-tests, after confirming normal distribution using the Shapiro-Wilk test. PDOs were classified as sensitive if the normalized GR_{AUC} of the FpOx screen was below the lower tertile, based on the one-third clinical response rate to adjuvant FpOx in patients with ctDNA-based MRD after surgery.[4]

RNA sequencing of patient-derived organoids

PDOs were harvested for sequencing four days after passage and lysed in RLT-buffer (Qiagen) with 1% β-mercaptoethanol to isolate RNA using QiaSymphony SP (Qiagen). After quality check using an Agilent Bioanalyzer (RNA Integrity Number >7), libraries were prepared with the Truseq RNA Stranded PolyA kit and sequenced on an Illumina NextSeq2000 (1x50bp). Raw reads were preprocessed, aligned to GRCh38, and quantified. RNA sequencing was performed in two runs, for which batch correction was applied using *limma* (v3.60.0). Counts were vst-normalized for visualization using Uniform Manifold Approximation and Projection (UMAP) and principal component analysis (PCA). Subsequently, differential gene expression analysis was performed using *DESeq2* (v1.44.0) with correction for sequencing batch. Differentially expressed genes (DEGs) were defined as a |log2FoldChange|>1 and a p-value<0.05 after False

Discovery Rate (FDR) correction using the Benjamin-Hochberg method. In addition to DEGs between PDOs classified as resistant versus sensitive to FpOx, normalized gene counts were correlated with continuous GR_{AUC} using two-sided Spearman correlation tests. Furthermore, gene set enrichment analysis (GSEA) was performed, reporting normalized enrichment scores (NES) between resistant and sensitive PDOs with p-values. In addition to hallmark gene sets based on MSigDB[27], we assessed previously published single cell RNA sequencing signatures that are specific to epithelial cells[28,29].

Whole genome sequencing of patient-derived organoids

From the same harvest as the RNA, PDOs were frozen for shipment to the Hartwig Medical Foundation. DNA was isolated from the PDO pellets and sequenced at a depth of >30× with paired-end reads of 150 base pairs, using the Illumina NovaSeq6000 whole genome sequencing (WGS) platform. Sequencing data was analyzed on the Hartwig somatic calling pipeline (http://www.github.com/hartwigmedical/pipeline5), hosted on a Google Cloud platform (https://github.com/hartwigmedical/platinum), which enables executing the complete Hartwig pipeline in a single run. Reads were mapped to GRCh38 using BWA (v0.7.17). SAGE (v2.2) was used to call somatic single and multi-nucleotide variants and indels. The utilized tools and settings are described in more detail elsewhere (https://github.com/hartwigmedical/hmftools).[30,31] In the absence of blood as germline control, sequencing reads were mapped against the human reference genome GRCh38.86, using the UMCU Illumina Analysis Pipeline hosted in the nextflow environment (https://github.com/UMCUGenetics/NF-IAP). VCF files containing raw variants were annotated with *snpeff* (v4.3), dbSNP (v138.b37) and COSMIC (v95). Likely benign variants were filtered out based on rsid. Driver mutations were classified as moderate or high impact based on oncoKb or clinvar.

Clinical validation cohort

Because of the limited number of PDOs, all biomarkers correlating with FpOx response in PDOs were validated in a larger parallel clinical cohort with comparable stage and treatment (Figure 1). This clinical cohort has been described in greater detail in a separate publication.[4] In brief, 199 patients with pMMR stage III colon cancer treated with resection and adjuvant FpOx were selected from the Prospective Dutch CRC cohort (PLCRC). PLCRC provides the infrastructure and written informed consent for

the collection of clinical data, resection material and blood samples (METC 12-510, NCT02070146). From formalin-fixed paraffin-embedded (FFPE) resection tissue, which may contain both tumor cells and microenvironment, we macro dissected the tumor region before RNA and DNA isolation. Bulk exome RNA sequencing on FFPE tissue required correction for the quality metrics DV200 and cDNA library prep concentration, which were used as covariates in DESeq2.[32] In parallel to the GSEA in the PDO cohort, we reported NES between patients with and without recurrence in the validation cohort. For individual genes of interest based on the PDO results, normalized counts were compared between patients with and without recurrence in the clinical validation cohort using Mann-Whitney U tests. Key driver mutations were assessed based on targeted DNA sequencing, using the PGDx® elio™ tissue complete assay.[33] P-values<0.05 were considered statistically significant. All analyses were performed in R (v4.0.3).

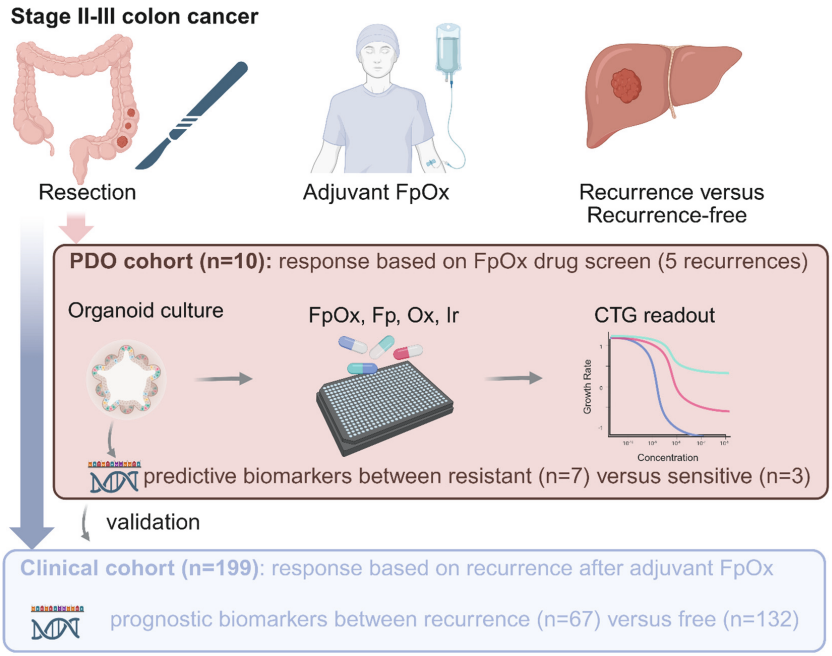


Figure 1: Study design: Stage II-III colon cancer tissue was obtained from resection, before adjuvant FpOx, to derive a PDO cohort and a parallel clinical cohort. The PDOs (n=10) were used to study drug screen response to FpOx, and to identify transcriptomic and genomic biomarkers with predictive value for response. Their prognostic value for recurrence was validated in the larger clinical cohort (n=199).
Fp, fluoropyrimidine; Ir, irinotecan; Ox, oxaliplatin; PDO, patient-derived organoid.

Results

Drug screens capture resistance to FpOx

PDOs were derived from five patients with and five patients without recurrence (characteristics in Supplementary Table 3), these groups were compared based on drug screen response. Dose-response curves are shown in Supplementary Figure 1 per PDO and per recurrence status, and the resulting GR_{AUC} is depicted in Figure 2A. The GR_{AUC} was higher in the group with compared to without recurrence, especially for doublet FpOx (mean GR_{AUC} 2.42 versus 1.50, p=0.035). A similar but less pronounced difference was observed for monotherapy screens with Fp (GR_{AUC} 2.76 versus 2.09, p=0.068) or Ox (GR_{AUC} 2.30 versus 1.67, p=0.085). In addition, PDOs showed a trend towards concurrent resistance to the alternative therapy Ir, although differential sensitivity to Ox and Ir was seen in some cases (Figure 2B). For instance, the most FpOx-resistant PDO (PERC1) corresponded to the patient with fastest recurrence after FpOx (11 months). This PDO was most sensitive to Ir, in line with this patient's clinical benefit from Ir after recurrence (progression-free survival (PFS) 14.5 months).

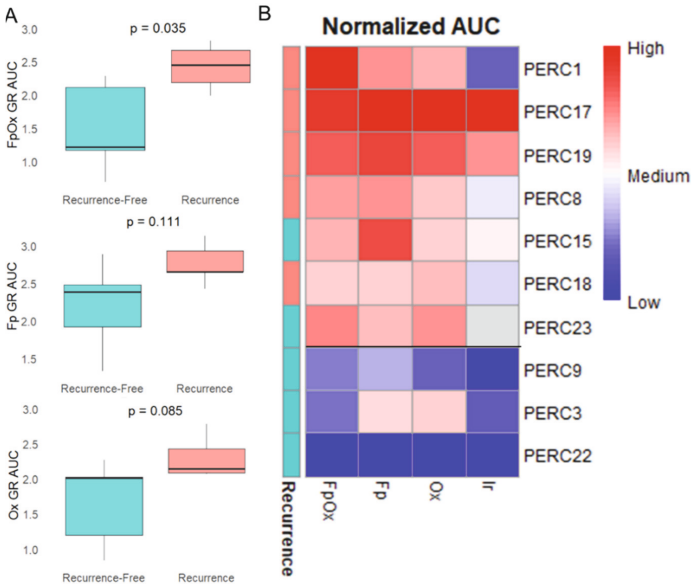


Figure 2: Drug screen results on PDO cohort. A) Sensitivity to FpOx, Fp and Ox summarized as GR_{AUC}, compared between patients without (n=5) and with (n=5) recurrence after adjuvant FpOx. B) Heatmap summarizing the normalized GR_{AUC} for Fpox, Fp, Ox and the alternative treatment Ir. Recurrence status is indicated in color. PERC9, 3 and 22 were classified as sensitive to the FpOx screen.
AUC, area under the curve; Fp, fluoropyrimidine; Ir, irinotecan; Ox, oxaliplatin.

The drug screen results were used to classify PDOs as resistant or sensitive to FpOx. The PDO's of all five patients with recurrence after adjuvant Fpox were resistant. The five PDOs from patients without recurrence, cured by either adjuvant FpOx or resection, showed a large range in chemosensitivity. Of them, two had a comparable GR_{AUC} to those with recurrence and were considered resistant, while only three were truly sensitive (Figure 2B; PERC3, 9, 22). Therefore, seven of the 10 PDOs were classified as resistant to FpOx, aligning with the two-thirds recurrence rate in patients with ctDNA-based MRD after surgery. Next, the resistant (n=7) and sensitive PDOs (n=3) were compared based on transcriptomic and genomic characteristics to identify biomarkers predictive of tumor-intrinsic FpOx response.

Mesenchymal genes correlate with FpOx resistance

Gene set enrichment analysis was performed between FpOx-resistant (n=7) versus FpOx-sensitive (n=3) PDOs and validated in the parallel clinical cohort between patients with (n=67) versus without recurrence (n=132) after adjuvant FpOx (Figure 3A, Supplementary Table 4). Hallmark gene sets linked to proliferation[34], like MYC, were lower in resistant PDOs (p=0.075) and in patients with recurrence in the clinical validation cohort (p=0.018). In contrast, resistant PDOs showed enriched angiogenesis (p=0.052) and EMT (p=0.62), which reached statistical significance in the clinical validation cohort (p=0.003; p=0.027, respectively).[12] This is in line with other enriched gene sets involved in EMT (PDO p=0.191; clinical p=0.003)[35], matrix remodeling (PDO p=0.398; clinical p=0.002), and associated signaling through transforming growth factor beta (TGF-β; PDO p=0.394; clinical p=0.072), platelet derived growth factor (PDGF; PDO p=0.056; clinical p=0.260) and fibroblast growth factor (FGF; PDO p=0.078; clinical p=0.124). In addition, two previously identified epithelial-specific gene sets of high-risk mesenchymal features were evaluated. In both resistant PDOs (p=0.061) and recurring patients (p=0.037) (Figure 3A), we observed an enrichment of Canellas' high-risk signature of epithelial genes (epiHR).[28] Additionally, Efremova's signature for epithelial regenerative cells (REC), which show upregulated EMT and colocalize with CAFs, correlated with resistance in the PDO cohort (p=0.006) and clinical cohort (p=0.162).[29]

Expression and mutation of individual genes to identify alternative treatment targets

In addition to gene sets, we looked at differential expression of individual genes between FpOx-resistant (n=7) versus sensitive (n=3) PDOs (Figure 3B; Supplementary Figure 2), and validated them between patients with (n=67) and without (n=132) recurrence (Supplementary Table 5). Of the 43 DEGs, *TM4SF20* had the highest fold change between PDO response groups (FC=56, FDR<0.001), and also had a significant positive Spearman correlation with the continuous FpOx GR_{AUC} per PDO (R=0.83, p=0.006).[36,37] *JUN* also signifcantly correlated with resistance on group-level (FC=6.00, FDR=0.031) and PDO-level (R=0.67, p=0.039), and was one of the core enriched genes in the gene sets corresponding to EMT, PDGF and FGFG. *JUN* encodes jun-c, which is part of the oncogenic transcription factor AP1 and is involved in TGF-β signaling.[38] The tumor suppressor *PHLPP1,* involved in proliferation and reduced EMT, was enriched in sensitive PDOs (FC=0.34, FDR=0.002; Spearman R=-0.67, p=0.039) and recurrence-free patients (FC=0.85, FDR=0.072; Mann-Whitney U p=0.027).[39,40] When looking for targetable genes, the enriched expression of *TACSTD2* in resistant PDOs (R=0.64, p=0.054) and recurring patients (p=0.024) may translate to TROP2 protein expression as target of novel antibody drug conjugates.[41-43]

Also on genomic level, we searched for driver mutations with predictive value for chemoresistance or targetability for alternative treatment. WGS identified no clear differences between FpOx-resistant and sensitive PDOs in this small cohort, and no significant differences between recurrence and recurrence-free patients in the clinical validation cohort (Supplementary Table 6). Most importantly, the PDOs not responding to FpOx did not harbor targetable mutations, like $KRAS^{G12C}$ and $BRAF^{V600E}$, that may direct additional treatment screens. Also in the clinical cohort, patients with recurrence after FpOx showed limited mutations that may inform alternative personalized treatment options in case of chemoresistance.[14]

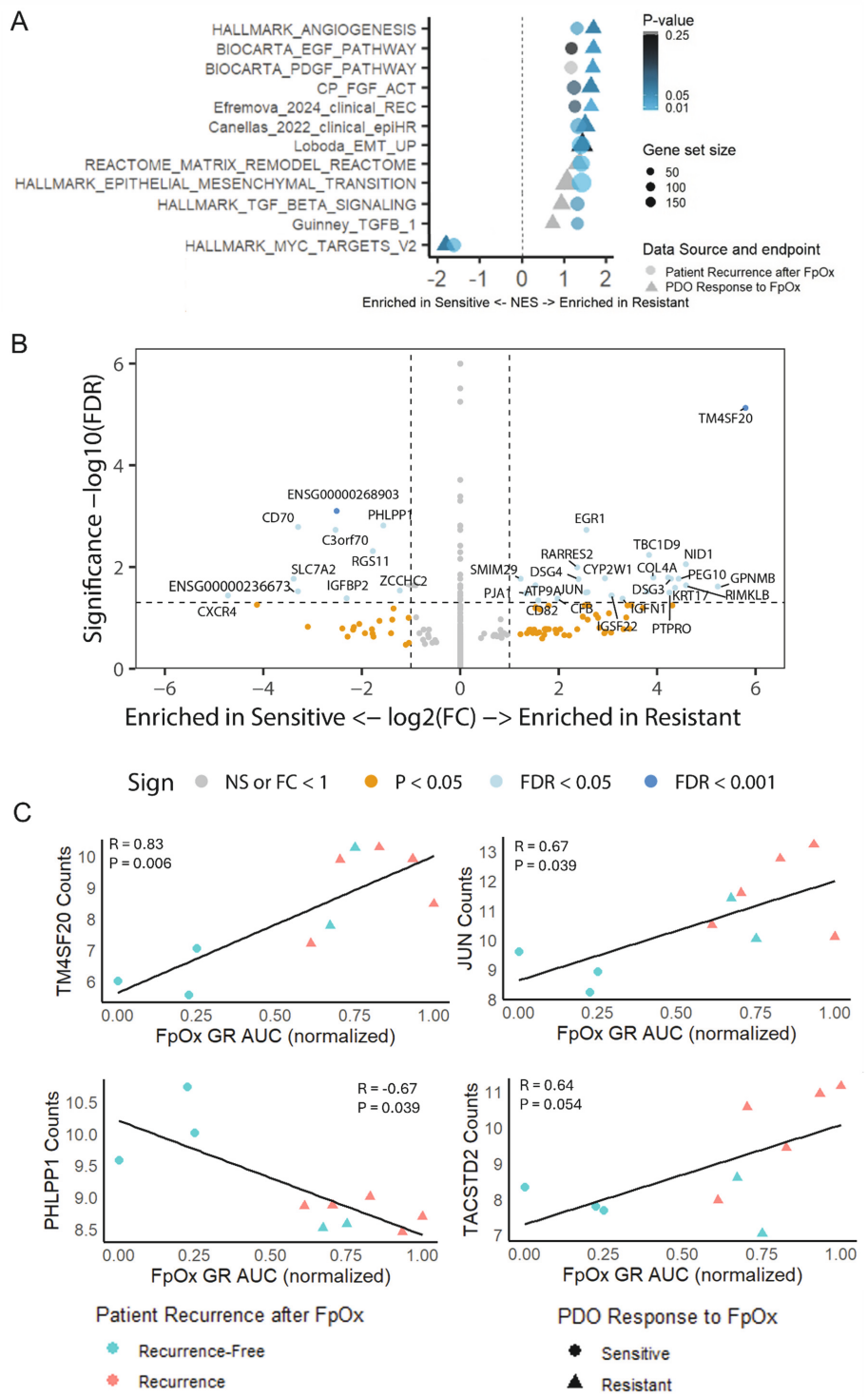

A
HALLMARK_ANGIOGENESIS
BIOCARTA_EGF_PATHWAY
BIOCARTA_PDGF_PATHWAY
CP_FGF_ACT
Efremova_2024_clinical_REC
Canellas_2022_clinical_eplHR
Loboda_EMT_UP
REACTOME_MATRIX_REMODEL_REACTOME
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
HALLMARK_TGF_BETA_SIGNALING
Guinney_TGFB_1
HALLMARK_MYC_TARGETS_V2
-2 -1 0 1 2
Enriched in Sensitive <- NES -> Enriched in Resistant
P-value
0.25
0.05
0.01
Gene set size
50
100
150
Data Source and endpoint
Patient Recurrence after FpOx
PDO Response to FpOx
B
Significance –log10(FDR)
Enriched in Sensitive <– log2(FC) –> Enriched in Resistant
TM4SF20
ENSG00000268903
CD70
PHLPP1
C3orf70
RGS11
SLC7A2
ENSG00000236673
IGFBP2
ZCCHC2
CXCR4
EGR1
SMIM29
RARRES2
DSG4
CYP2W1
TBC1D9
COL4A
NID1
PEG10
GPNMB
PJA1
ATP9A
JUN
DSG3
KRT17
RIMKLB
CD82
CPB
IGFN1
IGSF22
PTPRO
Sign
NS or FC < 1
P < 0.05
FDR < 0.05
FDR < 0.001
C
TM4SF20 Counts
R = 0.83
P = 0.006
FpOx GR AUC (normalized)
JUN Counts
R = 0.67
P = 0.039
FpOx GR AUC (normalized)
PHLPP1 Counts
R = -0.67
P = 0.039
FpOx GR AUC (normalized)
TACSTD2 Counts
R = 0.64
P = 0.054
FpOx GR AUC (normalized)
Patient Recurrence after FpOx
Recurrence-Free
Recurrence
PDO Response to FpOx
Sensitive
Resistant

Figure 3: RNA sequencing comparison in the PDO cohort between PDOs resistant (n=7) versus sensitive (n=3) to FpOx drug screen and in the clinical validation cohort between patients with (n=67) versus without recurrence (n=132). A) Gene set enrichment analysis, triangles indicate the PDO cohort and circles indicate the clinical validation cohort. B) Volcano plot of differentially expressed genes between resistant and sensitive PDOs. C) Correlation of normalized gene counts with continuous GR_{AUC} for FpOx drug screen. *AUC, area under the curve; FDR, false discovery rate; FpOx, fluoropyrimidine+oxaliplatin; GR, growth rate; NES, normalized enrichment score.*

Discussion

We investigated the potential of PDOs of stage II-III colon cancer to model chemoresistance and to identify biomarkers that can predict resistance. PDOs from patients with recurrence of disease were more resistant to drug screens with FpOx than PDOs from patients without recurrence. Most PDOs with resistance to FpOx were also resistant to the clinical alternative treatment irinotecan, highlighting the need for novel treatment avenues. Predominantly mesenchymal genes and signatures were overexpressed in PDOs with intrinsic resistance to FpOx drug screens, as well as in patients with recurrence after adjuvant FpOx in the clinical validation cohort.

We first showed that PDOs of primary colon cancer can capture tumor cell-intrinsic resistance to FpOx, using the drug screen optimization strategy published before.[23] PDOs from patients with recurrence after adjuvant FpOx were more resistant to FpOx. PDOs from patients without recurrence showed a larger variation in FpOx response, possibly because they were already cured by surgery alone, irrespective of sensitivity to adjuvant FpOx.[3] This indirect readout of clinical outcome, especially when no post-surgery ctDNA status is available, renders the adjuvant setting more difficult to study than the metastatic setting. Most prior studies correlated the more direct indicator of clinical response (RECIST or PFS) in metastatic CRC to drug screen sensitivity of PDOs derived from either metastatic lesions[22,23] and/or the primary tumor[18,19,24]. Smaller (sub)studies on adjuvant treatment of stage II-III colon cancer have shown correlation with heterogenous *in vitro* sensitivity to FpOx, in line with our results.[44]

After defining intrinsically chemoresistant PDO models, we aimed to find biomarkers for resistance. Chemoresistance was characterized by upregulated EMT, TGF-β signaling, and single-cell RNA sequencing-based epithelial gene signatures indicative of interaction with stromal cells.[28,29] Although the PDO cohort was limited by the small sample size, we strengthened our findings by validating these mesenchymal

biomarkers in the resection tissue of a larger clinical cohort.[14] Comparisons to the clinical cohort were challenging, because the bulk exome sequencing of the resection tissue covered not only gene expression by the epithelial tumor cells like in the PDOs, but also the TME. Nevertheless, we were able to validate some key findings in both cohorts, supporting that the mesenchymal biomarkers found to be of main prognostic value in the clinical cohort are not solely induced by TME but have a robust tumor-intrinsic role in chemoresistance.

In addition to modelling resistance to FpOx, PDOs offer potential to search for novel targets for therapy. Novel treatment is crucial, as alternatives like irinotecan[45] and cetuximab[46] have failed to improve outcome compared to FpOx in adjuvant studies. PDOs may provide a solution, as they show sensitivity to drugs that target tumor-specific DNA mutations[17,20,47]. However, mutations that guide targeted treatment in metastatic CRC, like *KRAS*^{G12C} [48] and *BRAF*^{V600E} [49], were absent in our resistant PDOs and low in the clinical validation cohort. On transcriptomic level, *TACSTD2* was a promising finding, as the expression of this gene and the encoded protein TROP2 are associated with cancer cell invasion[50] and worse overall and relapse-free survival in colon cancer[51,52]. TROP2 may be targetable with the antibody-drug conjugate sacituzumab-govitecan[42] or datopatomab-deruxtecan[43]. Other treatment targets may be provided by the modestly enriched signaling pathways TGF-β (i.a. galunisertib[53]), PDGF (i.a. imatinib[54]) and FGF (i.a. pemigatinib[55]). To further study the impact of TME on chemoresistance and potential targetability, future studies may focus on co-cultures of epithelial tumor cells with fibroblasts and immune cells.[56,57]

In conclusion, we show that colon cancer PDOs capture resistance to chemotherapy and provide a promising model to identify biomarkers for treatment response. Biomarkers that are associated with poor response to standard FpOx, and constitute possible treatment avenues, include high expression of mesenchymal genes and *TACSTD2*. A parallel clinical cohort helped to validate the prognostic value of these biomarkers for recurrence and concomitantly underline the complementary value of PDOs to study predictive value for tumor-intrinsic chemoresistance. Ultimately, this approach may help to not only identify patients who are not cured by the current FpOx, but also to provide them with alternative personalized treatment options.

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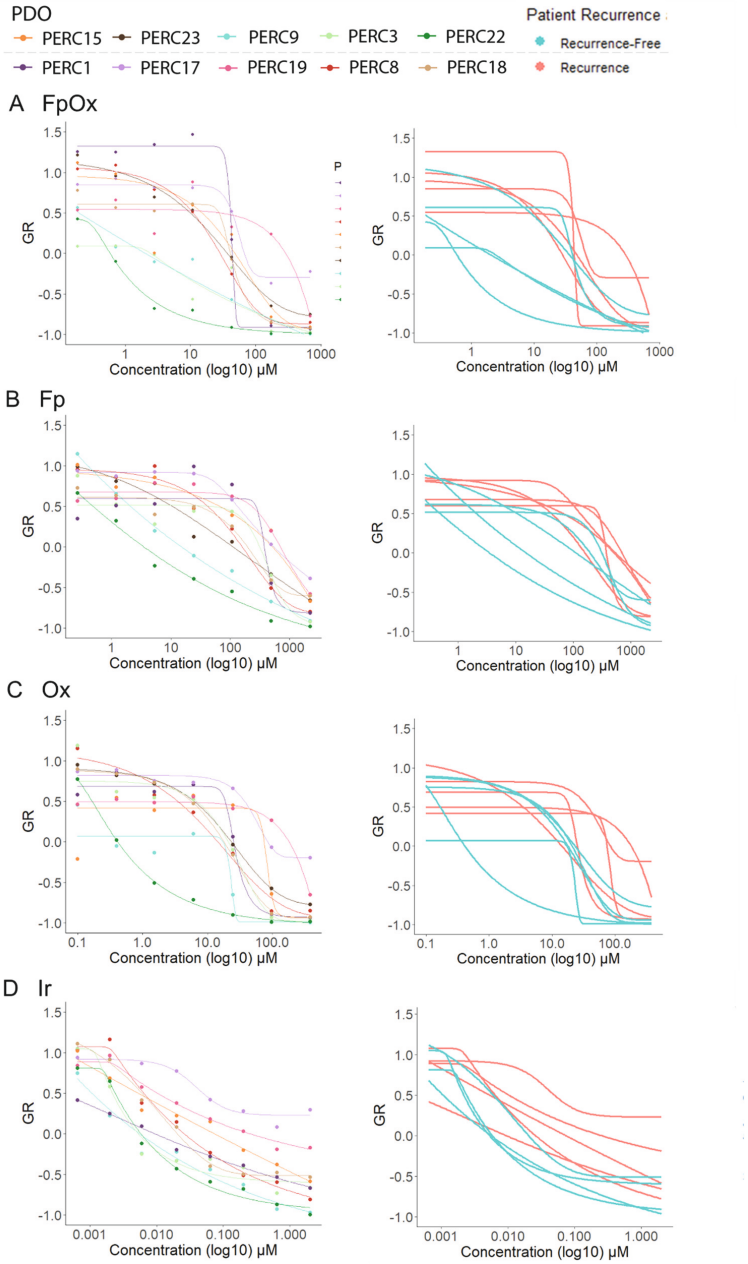
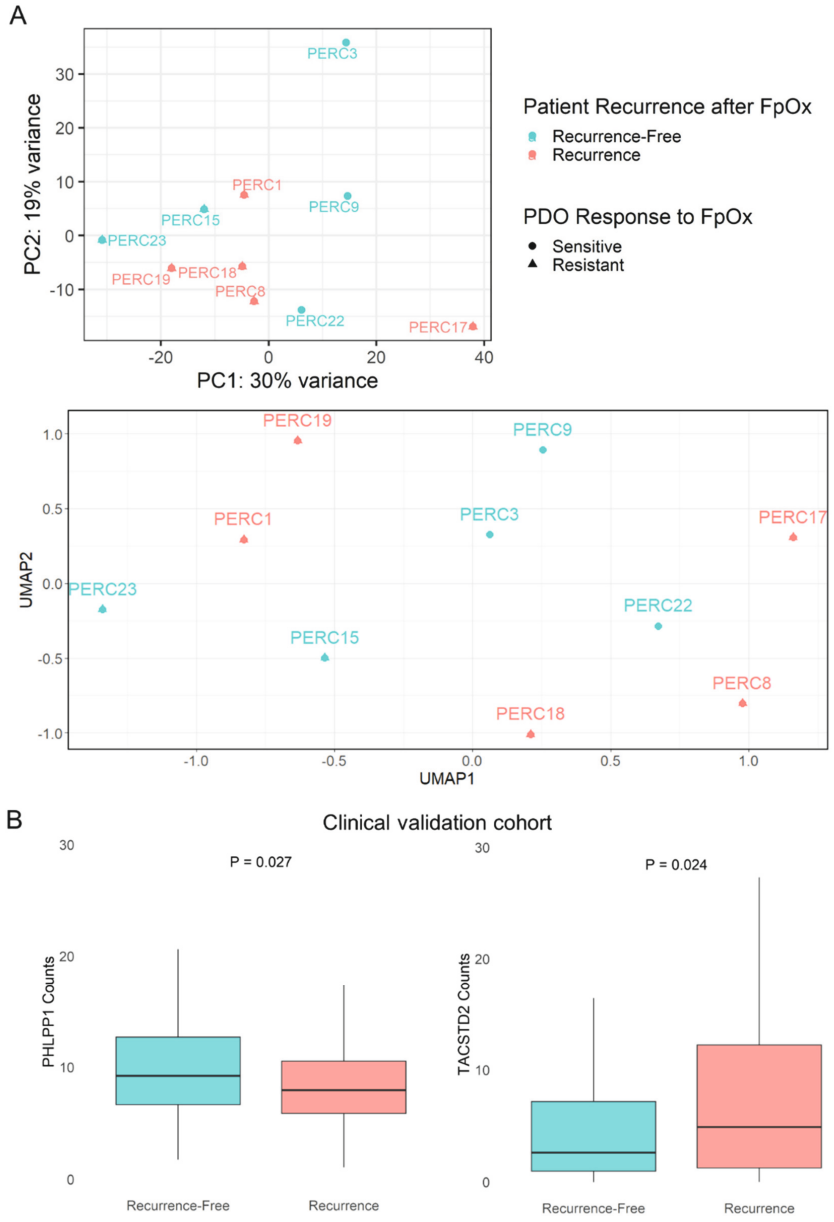
Supplementary materials

Supplementary Figure 1: Dose response curves per organoid and per recurrence status. The y-axis represents the GR metric from uninhibited growth (1) to cytostatis (0) to cytotoxicity (-1). Drugs include A) FpOx combination screen, B) Fp monoscreen, C) Ox monoscreen, D) Ir monoscreen.
FpOx, fluoropyrimidine and oxalipaltin; GR, growth rate; Ir, irinotecan; µM, micromolar.



Supplementary Figure 2: RNA sequencing comparison between sensitive and resistant PDOs. A) UMAP and PCA of PDO samples (n=10), colored by patient recurrence (n=5) and symbol by PDO resistance (n=7). B) Validation of differentially expressed genes from PDO cohort in the clinical validation cohort, counts presented per recurrence status and tested using Mann-Whitney U tests.
FpOx, fluoropyrimidine and oxaliplatin; PC, principal component; PDO, patient-derived organoid; UMAP, uniform manifold approximation and projection.



Supplementary Table 1: Organoid culturing medium and screening medium.

Component	Supplier	Culturing	Screening
Advanced DMEM/F12	Gibco, #12634028	Basis	Basis
Hepes	Santa Cruz Biotechnology, #SC-300789	10 mM	10 mM
Penicillin/Streptomycin	Gibco, #15070063	50 u/mL	50 u/mL
GlutaMAX	Gibco, #35050-038	2 mM	2 mM
A83-01 ALK inhibitor	MedChemExpress, #HY-10432	500 nM	500 nM
B27 without vitamin A	Gibco, #11530536	1:50	1:50
human recombinant EGF	Sigma-Aldrich, # E9644	50 ng/mL	50 ng/mL
recombinant human FGF-basic	eproTech, #100-18B	50 ng/ml	50 ng/ml
human recombinant IGF-I	BioLegend, #590908	100 ng/ml	100 ng/ml
N-acetyl-cysteine	Sigma-Aldrich, #A9165	1.25 mM	-
Noggin-conditioned medium UPE		100 ng/ml	100 ng/ml
recombinant human R-spondin-3	conditioned medium	1:5	1:5
Wnt surrogate	U-protein Express, #N001	0.05 nM	0.05 nM
Y27632 Rhokinase	MedChemExpress, #HY-10583	10 µM	-

Supplementary Table 2: Organoid drug screen layout: Per plate, four organoid lines are given the same drug concentration (µM) and volume (nL).*Conc, concentration; Fp, fluoropyrimidine; ir, irinotecan; navi, navitoclax; ox, oxaliplatin; vol, volume.*

Well	Drug	Conc_Fp	Ox	Ir	Navi	Vol_Fp	Ox	Ir	Navi	DMSO	Tween
A01	Ir			1.996				40		40	
A02	Ir			0.639				12.8		67.6	
A03	Ir			0.200				4		76	
A04	Ir			0.064				1.28		78.8	
A05	Ir			0.020				0.4		79.6	
A06	Ir			0.006				0.12		80	
A07	Ir			0.002				0.04		80	
A08	Ir			0.001				0.013		80	
A09	Tween										1682
A10	Fp	2212				922					760
A11	Fp	490				204					1478
A12	Fp	107				44					1637
A13	Fp	24				10					1673
A14	Fp	5.304				2.211					1680
A15	Fp	1.188				0.495					1682
A16	Fp	0.264				0.11					1682
A17	Tween										1682
A18	Ox		379				1397				285
A19	Ox		96				353				1328
A20	Ox		24				89				1593
A21	Ox		6.08				22				1660
A22	Ox		1.522				5.6				1677
A23	Ox		0.395				1.452				1681
A24	Ox		0.099				0.363				1682
B01	Ir			1.996				40		40	
B02	Ir			0.639				12.8		67.6	
B03	Ir			0.200				4		76	
B04	Ir			0.064				1.28		78.8	
B05	Ir			0.020				0.4		79.6	
B06	Ir			0.006				0.12		80	
B07	Ir			0.002				0.04		80	
B08	Ir			0.001				0.013		80	
B09	Tween										1682
B10	Fp	2212				922					760
B11	Fp	490				204					1478
B12	Fp	107				44					1637

Well	Drug	Conc_Fp	Ox	Ir	Navi	Vol_Fp	Ox	Ir	Navi	DMSO	Tween
B13	Fp	24				10					1673
B14	Fp	5.304				2.211					1680
B15	Fp	1.188				0.495					1682
B16	Fp	0.264				0.11					1682
B17	Tween	2212									1682
B18	Ox		379				1397				285
B19	Ox		96				353				1328
B20	Ox		24				89				1593
B21	Ox		6.089				22				1660
B22	Ox		1.522				5.6				1677
B23	Ox		0.395				1.452				1681
B24	Ox		0.099				0.363				1682
C01	Ir			1.996				40		40	
C02	Ir			0.639				12.8		67.6	
C03	Ir			0.200				4		76	
C04	Ir			0.064				1.28		78.8	
C05	Ir			0.020				0.4		79.6	
C06	Ir			0.006				0.12		80	
C07	Ir			0.002				0.04		80	
C08	Ir			0.001				0.013		80	
C09	Tween										1682
C10	Fp	2212				922					760
C11	Fp	490				204					1478
C12	Fp	107				44					1637
C13	Fp	24				10					1673
C14	Fp	5.304				2.211					1680
C15	Fp	1.188				0.495					1682
C16	Fp	0.264				0.11					1682
C17	Tween										1682
C18	Ox	379	379	379			1397				285
C19	Ox	96	96	96			353				1328
C20	Ox	24	24	24			89				1593
C21	Ox	6.089	6.089	6.089			22				1660
C22	Ox	1.522	1.522	1.522			5.6				1677
C23	Ox	0.395	0.395	0.395			1.452				1681
C24	Ox	0.099	0.099	0.099			0.363				1682
D01	Navi				19.96				80		
D02	Navi				19.96				80		
D03	Navi				19.96				80		
D04	Navi				19.96				80		
D05					40080		80			0.002	
D06					40080		80			0.002	
D07					40080		80			0.002	
D08					40080		80			0.002	
D09	Tween										1682
D10	Fp_Ox	683	379			285	1397				
D11	Fp_Ox	173	96			72	353				1257
D12	Fp_Ox	42	24			17	89				1575
D13	Fp_Ox	10	6.089			4.48	22				1656
D14	Fp_Ox	2.797	1.522			1.166	5.6				1676
D15	Fp_Ox	0.713	0.395			0.297	1.452				1681
D16	Fp_Ox	0.185	0.099			0.077	0.363				1682
D17	Tween										1682
D18	Fp_Ox	683	379			285	1397				
D19	Fp_Ox	173	96			72	353				1257
D20	Fp_Ox	42	24			17	89				1575
D21	Fp_Ox	10	6.089			4.48	22				1656
D22	Fp_Ox	2.797	1.522			1.166	5.6				1676
D23	Fp_Ox	0.713	0.395			0.297	1.452				1681
D24	Fp_Ox	0.185	0.099			0.077	0.363				1682

Supplementary Table 3: Clinical and pathological data of the organoid cohort.*AC, adenocarcinoma; ACT, adjuvant chemotherapy; diff, differentiation; FpOx; fluoro-pyrimidine+oxaliplatin; inv, invasion; muc, mucinous; PDO, patient-derived organoid.*

PDO	Sex	Age	pT	pN	Tumor side	Histology	Diff	Lymph Inv	Vasc Inv	ACT	Cycles	Recurrence (months)	Resistance PDO FpOx	Mutation status
PERC1	Female	65	T4	N1	Right	AC	Good	Yes	No	FpOx	4	Liver(11)	Resistant	NRASQ61L, SMAD4 G510* , APC Q767*, TP53 R175H
PERC17	Female	64	T3	N2	Left	Muc	Good	No	No	FpOx	8	Liver(42)	Resistant	
PERC19	Male	82	T4	N2	Left	AC	Good	No	Yes	Fp	3	Local42)	Resistant	TP53 Y107D
PERC8	Male	69	T3	N0	Right	AC	Poor	No	No	FpOx	8	Lung(38)	Resistant	KRASG12V, TP53 R273C, PIK3CA E542K
PERC15	Female	67	T4	N2	Left	AC	Poor	No	No	FpOx	8	Free(106)	Resistant	
PERC18	Female	75	T3	N1	Left	AC	Good	Yes	No	Fp	8	Liver(21)	Resistant	APC R1450*, TP53 R273C
PERC23	Female	65	T3	N1	Left	AC	Poor	No	Yes	FpOx	8	Free(99)	Resistant	APC Q1328*, TP53 R273H, ALK I1461V
PERC9	Female	71	T3	N1	Left	AC	Poor	No	Yes	FpOx	1	Free(50)	Sensitive	KRASG13D, APC E153*
PERC3	Female	67	T3	N1	Right	Muc	Good	No	Yes	FpOx	2	Free(38)	Sensitive	NRAS Y64S, BRAFV600E, FGFR3 A570V , TP53 R248W, PIK3CA E453del
PERC22	Male	70	T3	N1	Right	AC	Good	Yes	Yes	FpOx	8	Free(99)	Sensitive	APC Q1611*

Supplementary Table 4: Normalized enrichment scores of gene sets across PDO resistance and patient recurrence. *FDR, false discovery rate; PDO, patient-derived organoid; NES, normalized enrichment score.*

		PDO				Clinical validation cohort			
Description	setSize	NES	pvalue	FDR	qvalue	NES	pvalue	FDR	qvalue
ADIPOGENESIS	191	1.244	0.091	0.600	0.600	0.866	0.745	0.896	0.736
ALLOGRAFT_REJECTION	175	1.273	0.506	0.716	0.716	0.824	0.798	0.904	0.742
ANDROGEN_RESPONSE	98	1.476	0.151	0.600	0.600	1.030	0.458	0.849	0.697
ANGIOGENESIS	30	1.670	0.079	0.600	0.600	1.335	0.046	0.208	0.171
APICAL_JUNCTION	187	1.144	0.636	0.716	0.716	1.215	0.121	0.361	0.297
APICAL_SURFACE	39	-1.175	0.228	0.600	0.600	0.953	0.611	0.888	0.729
APOPTOSIS	155	1.313	0.390	0.636	0.636	0.925	0.663	0.888	0.729
BILE_ACID_METABOLISM	108	-1.785	0.027	0.600	0.600	0.800	0.813	0.904	0.742
CHOLESTEROL_HOMEOSTASIS	72	-0.949	0.455	0.690	0.690	-0.987	0.436	0.839	0.688
COAGULATION	124	0.727	0.580	0.716	0.716	1.231	0.130	0.361	0.297
COMPLEMENT	179	1.261	0.456	0.690	0.690	1.274	0.057	0.217	0.179
DNA_REPAIR	149	-0.696	0.779	0.811	0.811	-1.065	0.392	0.828	0.680
E2F_TARGETS	200	-0.684	0.762	0.810	0.810	-1.709	0.000	0.002	0.001
EPITHELIAL_MESENCHYMAL_TRANSITION	174	1.084	0.622	0.716	0.716	1.407	0.003	0.042	0.034
ESTROGEN_RESPONSE_EARLY	191	-1.383	0.237	0.600	0.600	1.058	0.398	0.828	0.680
ESTROGEN_RESPONSE_LATE	192	-1.719	0.186	0.600	0.600	0.926	0.674	0.888	0.729
FATTY_ACID_METABOLISM	150	-0.991	0.291	0.600	0.600	-1.080	0.353	0.802	0.659
G2M_CHECKPOINT	199	-0.942	0.337	0.601	0.601	-1.416	0.036	0.180	0.148
GLYCOLYSIS	197	-1.718	0.168	0.600	0.600	0.920	0.680	0.888	0.729
HEDGEHOG_SIGNALING	31	0.858	0.572	0.716	0.716	1.266	0.122	0.361	0.297
HEME_METABOLISM	186	1.245	0.490	0.716	0.716	1.008	0.508	0.888	0.729
HYPOXIA	191	-1.139	0.257	0.600	0.600	1.345	0.013	0.081	0.067
IL2_STAT5_SIGNALING	190	-1.714	0.171	0.600	0.600	0.838	0.784	0.904	0.742
IL6_JAK_STAT3_SIGNALING	78	1.570	0.102	0.600	0.600	0.913	0.657	0.888	0.729
INFLAMMATORY_RESPONSE	180	-1.307	0.238	0.600	0.600	0.881	0.728	0.896	0.736
INTERFERON_ALPHA_RESP	94	1.485	0.380	0.636	0.636	-1.154	0.291	0.694	0.570
INTERFERON_GAMMA_RESP	188	1.248	0.567	0.716	0.716	-0.847	0.667	0.888	0.729
KRAS_SIGNALING_DN	165	-1.751	0.063	0.600	0.600	1.395	0.006	0.051	0.042
KRAS_SIGNALING_UP	174	0.959	0.635	0.716	0.716	1.285	0.052	0.217	0.178
MITOTIC_SPINDLE	196	1.229	0.632	0.716	0.716	0.771	0.859	0.914	0.751
MTORC1_SIGNALING	197	-1.718	0.168	0.600	0.600	-0.906	0.545	0.888	0.729
MYC_TARGETS_V1	200	-0.977	0.318	0.600	0.600	-1.671	0.000	0.003	0.003
MYC_TARGETS_V2	58	-1.785	0.076	0.600	0.600	-1.567	0.018	0.100	0.082
MYOGENESIS	181	1.259	0.157	0.600	0.600	1.382	0.003	0.042	0.034
NOTCH_SIGNALING	32	-1.105	0.324	0.600	0.600	1.195	0.222	0.584	0.480
OXIDATIVE_PHOSPHORYL	199	-1.168	0.261	0.600	0.600	-1.499	0.007	0.051	0.042
P53_PATHWAY	188	1.249	0.300	0.600	0.600	0.866	0.753	0.896	0.736
PANCREAS_BETA_CELLS	33	0.631	0.932	0.932	0.932	1.400	0.004	0.042	0.034
PEROXISOME	103	-1.797	0.098	0.600	0.600	-1.011	0.414	0.829	0.681
PI3K_AKT_MTOR_SIGNALING	103	-1.798	0.002	0.077	0.077	0.602	0.979	0.979	0.804
PROTEIN_SECRETION	96	0.681	0.644	0.716	0.716	0.924	0.650	0.888	0.729
REACTIVE_OXYGEN_SPECIES	49	0.667	0.808	0.824	0.824	-0.856	0.693	0.888	0.729
SPERMATOGENESIS	118	-1.006	0.299	0.600	0.600	0.761	0.856	0.914	0.751
TGF_BETA_SIGNALING	53	0.936	0.394	0.636	0.636	1.311	0.073	0.259	0.213
TNFA_SIGNALING_VIA_NFKB	187	1.246	0.275	0.600	0.600	1.149	0.234	0.585	0.480
UNFOLDED_PROTEIN_RESP	113	-0.832	0.568	0.716	0.716	-0.681	0.950	0.970	0.796
UV_RESPONSE_DN	138	1.348	0.252	0.600	0.600	1.235	0.126	0.361	0.297
UV_RESPONSE_UP	152	0.764	0.622	0.716	0.716	-0.884	0.590	0.888	0.729
WNT_BETA_CATENIN_SIGN	41	1.650	0.161	0.600	0.600	-0.884	0.568	0.888	0.729
XENOBIOTIC_METABOLISM	191	0.971	0.661	0.719	0.719	0.751	0.884	0.921	0.756
Basak_2014_mKi67	58	-1.157	0.213	0.591	0.565	-1.556	0.014	0.168	0.144
BIOCARTA_EGF_PATHWAY	27	1.690	0.056	0.189	0.189	1.202	0.256	0.269	0.177
BIOCARTA_PDGF_PATHWAY	26	1.683	0.056	0.189	0.189	1.199	0.269	0.269	0.177
BIOCARTA_INTEGRIN	25	1.675	0.109	0.475	0.454	1.162	0.313	0.495	0.425
BIOCARTA_NFKB	22	1.362	0.123	0.475	0.454	1.005	0.515	0.715	0.613
Canellas_2022_clinical_coreHRC	83	0.994	0.410	0.686	0.655	1.338	0.033	0.218	0.187
Canellas_2022_clinical_epiHR	86	1.513	0.061	0.402	0.384	1.339	0.037	0.218	0.187
CP_FGF_ACT	49	1.643	0.078	0.421	0.402	1.243	0.163	0.367	0.315
CP_IGF1R	29	1.714	0.121	0.475	0.454	1.182	0.255	0.433	0.372
CP_MYC_TARGETS	43	1.679	0.006	0.157	0.150	-1.275	0.208	0.395	0.338
CP_NOTCH	55	0.592	0.940	0.940	0.898	1.320	0.074	0.284	0.243
CP_SHH	47	0.773	0.619	0.803	0.767	0.687	0.888	0.946	0.811
CP_VEGF_VEGFR	8	-1.196	0.267	0.616	0.588	1.268	0.152	0.367	0.315
Efremova_2024_Colonocyte	16	-1.113	0.353	0.661	0.631	0.765	0.802	0.874	0.750
Efremova_2024_Enteroendocrine	20	-1.410	0.055	0.402	0.384	1.392	0.004	0.086	0.073
Efremova_2024_Goblet	18	-1.265	0.153	0.531	0.508	1.319	0.077	0.284	0.243
Efremova_2024_HLAhigh	20	1.642	0.069	0.402	0.384	-1.537	0.025	0.210	0.180
Efremova_2024_Hypoxia	15	-1.013	0.463	0.711	0.679	1.222	0.207	0.395	0.338
Efremova_2024_Intermediate	17	1.201	0.242	0.610	0.583	-1.207	0.252	0.433	0.372

Efremova_2024_iREC	21	1.648	0.070	0.402	0.384	-1.536	0.038	0.218	0.187
Efremova_2024_REC	18	1.633	0.006	0.157	0.150	1.260	0.162	0.367	0.315
Efremova_2024_Stem	22	0.967	0.470	0.711	0.679	-1.583	0.011	0.156	0.134
Efremova_2024_StemNOTUM	19	1.634	0.004	0.157	0.150	-1.388	0.135	0.366	0.314
Efremova_2024_TA1	21	-1.439	0.048	0.402	0.384	1.039	0.489	0.698	0.598
Efremova_2024_TA2	5	-1.009	0.546	0.750	0.716	-1.328	0.123	0.360	0.309
Efremova_2024_UPR	19	-0.985	0.502	0.724	0.691	1.219	0.223	0.403	0.346
Guinney_TGFB_1	26	0.737	0.756	0.844	0.806	1.340	0.057	0.261	0.224
Guinney_TGFB_2	86	1.513	0.070	0.402	0.384	1.261	0.115	0.350	0.300
Harmonizome_TGF beta signalling	79	1.530	0.127	0.475	0.454	1.144	0.292	0.480	0.412
Harmonizone_cholesterol	11	-0.946	0.592	0.797	0.761	0.550	0.945	0.964	0.827
Harmonizone_JAK/STAT	13	-1.055	0.423	0.691	0.660	0.956	0.600	0.738	0.633
KEGG_CELL_CYCLE	76	0.712	0.621	0.803	0.767	-1.413	0.051	0.258	0.221
KEGG_COMPLEMENT_COAG	16	-0.716	0.845	0.863	0.824	1.331	0.059	0.261	0.224
KEGG_JAK_STAT	122	-0.743	0.693	0.830	0.792	-0.786	0.817	0.881	0.755
KEGG_PROTEASOME	16	-0.753	0.805	0.844	0.806	-1.357	0.140	0.366	0.314
KEGG_SHH	54	1.633	0.123	0.475	0.454	0.928	0.634	0.750	0.643
KEGG_TGFB	82	-0.724	0.806	0.844	0.806	1.233	0.158	0.367	0.315
Loboda_EMT_UP	112	1.447	0.191	0.591	0.565	1.418	0.003	0.061	0.053
Michels_APC-KO Protein	156	-0.747	0.672	0.826	0.789	-0.751	0.897	0.946	0.811
Michels_APC-KO sig	95	0.962	0.442	0.691	0.660	0.997	0.521	0.715	0.613
Michels_Wnt sig	136	-1.723	0.005	0.157	0.150	-0.932	0.556	0.738	0.633
Michels_Wnt-Protein	35	-1.057	0.376	0.661	0.631	-1.409	0.140	0.366	0.314
Oncotype	5	0.760	0.814	0.844	0.806	1.213	0.216	0.403	0.346
REACTOME_MATRIX_REMODEL	97	1.365	0.398	0.677	0.646	1.419	0.002	0.055	0.048
REACTOME_NOTCH	94	1.494	0.134	0.481	0.460	0.956	0.599	0.738	0.633
Zeller_MYC_TARGETS	47	-0.728	0.792	0.844	0.806	-1.367	0.158	0.367	0.315

Supplementary Table 5: Differentially expressed genes across PDO resistance and patient recurrence. Showing genes meeting the threshold (FoldChange >2, FDR adjusted p-value <0.05), results of the remaining genes are available online.
FC, fold change; FDR, false discovery rate; PDO, patient-derived organoid; SE, standard error.

	PDO cohort					Clinical validation cohort				
gene	**FC**	**log2FC**	**lfcSE**	**pvalue**	**FDR**	**FC**	**log2FC**	**lfcSE**	**pvalue**	**FDR**
TM4SF20	55.574	*5.796*	1.480	0.000	0.000	0.999	-0.002	0.085	0.923	0.982
EDA2R	0.010	-6.597	1.253	0.000	0.000	1.011	0.016	0.084	0.590	0.852
ZNF331	94.450	6.561	1.450	0.000	0.000	1.049	0.069	0.099	0.150	0.501
VWDE	111.725	6.804	1.531	0.000	0.000	0.561	-0.835	0.457	0.001	0.041
ENSG00000268903	0.175	-2.511	0.581	0.000	0.001	1.000	0.000	0.083	0.971	0.993
PHLPP1	0.339	-1.560	0.366	0.000	0.002	0.849	-0.236	0.118	0.004	0.072
CD70	0.102	-3.294	0.854	0.000	0.002	0.969	-0.046	0.098	0.117	0.447
C3orf70	0.173	-2.534	0.645	0.000	0.002	1.077	0.107	0.142	0.053	0.307
EGR1	5.922	2.566	0.670	0.000	0.002	1.094	0.130	0.146	0.037	0.259
RGS11	0.292	-1.777	0.455	0.000	0.005	1.056	0.078	0.120	0.076	0.365
TBC1D9	14.284	3.836	1.269	0.000	0.006	1.065	0.091	0.103	0.093	0.403
NID1	23.970	4.583	1.483	0.000	0.009	1.042	0.060	0.075	0.169	0.527
RARRES2	5.199	2.378	0.681	0.000	0.010	1.105	0.145	0.124	0.029	0.227
HDGFL3	94.021	6.555	2.081	0.000	0.011	1.008	0.011	0.078	0.772	0.931
KRT31	242.591	7.922	3.351	0.000	0.013	1.000	0.000	0.088	0.925	0.983
COL4A1	15.172	3.923	1.262	0.000	0.016	1.156	0.209	0.112	0.007	0.106
SDR16C5	7.677	2.940	0.992	0.000	0.017	1.039	0.055	0.109	0.058	0.320
DSG3	19.460	4.282	1.906	0.000	0.017	1.024	0.035	0.094	0.132	0.472
SLC7A2	0.096	-3.383	0.947	0.000	0.017	1.047	0.066	0.109	0.107	0.427
PEG10	21.657	4.437	1.623	0.000	0.017	0.985	-0.022	0.088	0.347	0.706
SMIM29	2.343	1.228	0.387	0.000	0.017	1.059	0.083	0.091	0.115	0.444

DSG4	5.277	2.400	0.762	0.000	0.017	0.978	-0.032	0.088	0.326	0.689
ALDH1A2	72.551	6.181	2.119	0.000	0.023	1.071	0.098	0.193	0.009	0.120
ATP9A	2.884	1.528	0.518	0.000	0.023	1.028	0.039	0.073	0.389	0.735
CCNB1IP1	0.534	-0.906	0.276	0.000	0.023	1.037	0.053	0.082	0.264	0.638
RIMKLB	24.037	4.587	1.848	0.000	0.023	1.461	0.547	0.180	0.000	0.010
GPNMB	37.524	5.230	1.937	0.000	0.024	1.069	0.097	0.114	0.077	0.368
KRT17	20.595	4.364	1.570	0.000	0.026	1.008	0.012	0.085	0.611	0.863
ZCCHC2	0.427	-1.227	0.385	0.000	0.029	0.997	-0.004	0.069	0.881	0.969
JUN	6.005	2.586	0.969	0.000	0.031	1.055	0.077	0.091	0.133	0.474
PTPRO	19.039	4.251	1.936	0.000	0.032	0.967	-0.049	0.100	0.129	0.466
PJA1	2.523	1.335	0.502	0.000	0.033	0.982	-0.026	0.073	0.495	0.803
CXCR4	0.038	-4.717	1.531	0.000	0.036	1.078	0.109	0.125	0.069	0.349
CYP2W1	8.399	3.070	1.259	0.000	0.036	0.999	-0.001	0.085	0.988	0.997
IGSF22	8.443	3.078	1.013	0.000	0.036	0.968	-0.046	0.102	0.071	0.354
IGFBP2	0.202	-2.305	0.773	0.000	0.041	1.008	0.011	0.082	0.732	0.915
CFB	3.929	1.974	0.765	0.000	0.042	0.978	-0.033	0.088	0.315	0.682
IGFN1	9.837	3.298	1.419	0.000	0.042	1.359	0.442	0.306	0.004	0.082
CD82	2.998	1.584	0.623	0.000	0.045	1.127	0.172	0.102	0.011	0.139
MUC5B	0.057	-4.130	1.431	0.000	0.056	1.010	0.015	0.086	0.513	0.813
COL6A2	19.812	4.308	1.817	0.000	0.057	1.205	0.269	0.168	0.008	0.112

Supplementary Table 6: Mutation status in driver genes and potentially targetable genes across PDO resistance and patient recurrence. *Mut, mutant; wt, wildtype.*

	PDO cohort		**Clinical validation cohort**	
	Sensitive (n=3)	Resistant (n=7)	Recur-Free (n=132)	Recurrence (n=67)
APC				
wt	1 (33%)	4 (57%)	108 (82)	50 (77%)
mut	2 (67%)	3 (43%)	23 (18%)	15 (23%)
TP53				
wt	3 (100%)	2 (29%)	40 (31%)	19 (29%)
mut	0 (0%)	5 (71%)	91 (69%)	46 (71%)
KRAS				
wt	2 (67%)	5 (71%)	70 (53%)	34 (51%)
mut	1 (33%)	2 (29%)	61 (47%)	33 (49%)
NRAS				
wt	2 (67%)	6 (86%)	127 (97%)	62 (95%)
mut	1 (33%)	1 (14%)	4 (3%)	3 (5%)
BRAF				
wt	2 (67%)	7 (100%)	122 (93%)	57 (85%)
mut	1 (33%)	0 (0%)	9 (7%)	10 (15%)
PIK3CA				
wt	2 (67%)	6 (86%)	105 (80%)	56 (86%)
mut	1 (33%)	1 (14%)	26 (20%)	9 (14%)
SMAD4				
wt	3 (100%)	6 (86%)	120 (92%)	55 (85%)
mut	0 (0%)	1 (14%)	11 (8%)	10 (15%)

8

General discussion

Colorectal cancer (CRC) is a major health concern. Treatment recommendations are based on T/N stage and mismatch repair (MMR) status. For high-risk stage II and stage III colon cancer (CC), adjuvant chemotherapy (ACT) has consisted for over two decades of a fluoropyrimidine with oxaliplatin (CAPOX/FOLFOX). This one-size-fits-all approach does not benefit all patients, highlighting the need to move to personalized treatment. The main challenges are to identify patients who are over- and undertreated with current standard of care, and to respectively develop de-escalation and escalation strategies. This thesis aims to identify factors that are prognostic of disease recurrence and predictive of treatment response, using real-world data and biomarkers.

Nationwide registry to describe the general population and complement trials

The gold standard in research is a randomized controlled trial (RCT), randomizing patients to a new intervention or standard of care. Since confounding factors are expected to distribute equally, any differences in overall survival (OS) or disease-free survival (DFS) can be attributed to the difference in treatment. RCTs usually apply strict inclusion criteria regarding patient fitness and disease burden. Therefore, the observed treatment efficacy in trials does not always transfer to effectiveness in the general population.[1] In addition, RCTs may not be feasible when one treatment arm is anticipated to yield substantially better outcomes or when the disease is very rare. In these cases, real-world data (RWD) may provide a solution. The Netherlands Cancer Registry (NCR) includes all patients diagnosed with cancer, hence providing a complete overview of the patient population. We leveraged NCR data to assess the impact of trial-based guideline changes on clinical practice (chapter 2) and to provide context to single-arm intervention studies for rare molecular subgroups (chapter 3).

The most recent guideline change in high-risk stage II (pT4N0 with proficient MMR (pMMR)/microsatellite stability (MSS)) and stage III (pN+) CC includes the reduction of adjuvant CAPOX duration from six to three months, based on non-inferiority in the IDEA trials.[2,3] Non-inferiority was statistically not met in the subgroup with high-risk stage III (pT4/N2) CC, raising the question as to whether the observed OS difference (1%) should be considered clinically relevant. Due to fear of undertreatment, high-risk stage III CC is often treated with six months CAPOX internationally[4,5], while the Dutch guideline adopted three months CAPOX for all substages.[6] This provided the unique opportunity to demonstrate in Chapter 2 that there was no difference in OS between

six and three months CAPOX in all patients in the NCR, including high-risk stage III CC. Also unique was the access to patient-reported outcomes through the Prospective Dutch Colorectal Cancer (PLCRC) cohort, showing long-term improvement in peripheral neuropathy[2], quality of life[7] and workability[8]. Together, these findings complement the IDEA trials by providing real-world evidence to support three months of CAPOX in both low- and high-risk CC, helping to reduce overtreatment and toxicity.

Patient characteristics and survival in our population-based cohort in chapter 2 were comparable to the IDEA trials. The efficacy-effectiveness gap between RCT and RWD, as reported in studies in metastatic CRC (mCRC)[9,10], was not evident in our study and in prior adjuvant studies[11,12]. This may be attributable to the lower disease burden and thus less heterogeneity in disease-related complaints in non-metastatic CRC. In addition, the IDEA trials had a relatively pragmatic design and lenient inclusion criteria. Our findings on comparable OS after six versus three months ACT have been corroborated in two different studies using the province-wide Alberta Cancer Registry. One study compared patients treated before versus after the guideline change, as an intention-to-treat analysis of six versus three months (reply in Chapter 2a).[13,14] The other study included only patients treated before the guideline change, comparing those receiving six months to those receiving three months in a per-protocol analysis. OS was worse after three months CAPOX in the naïve analysis, but this was not the case when emulating the IDEAL trial's inclusion criteria and accounting for time-varying confounding factors.[15] Such trial emulations can help infer causal treatment effects, when treatment allocation is not randomized between two observational arms. Nevertheless, observational data should be interpreted with caution as information on the planned treatment and (reasons for) deviations is usually not well documented.[16,17]

RCTs may also be challenging when moving to smaller specific tumor subtypes, causing researchers to resort to single-arm intervention trials. An example is rectal cancer (RC) with deficient MMR (dMMR; 2-5%), showing 100% clinical complete response without the need for additional surgery in a recent trial on immune checkpoint inhibition (ICI, anti- programmed cell death protein 1 (PD-1) dostarlimab).[18,19] The study did not contain a control arm, complicating causal inferences on the benefit derived from ICI compared to standard of care. After the promising results in this and other single-arm ICI trials[20,21], it may not be considered ethical to prospectively

randomize dMMR RC patients between ICI and a control arm that is expected to be inferior. In such cases, RWD registries can provide an external or hybrid[22] historical control arm[16,17,23]. We aim this in chapter 3, based on the largest cohort of dMMR RC to date. We show that dMMR (2.3%) compared to pMMR stage II/III RC was associated with better outcomes after standard of care. This included a higher rate of clinical complete response (dMMR 31% versus pMMR 12%) to neoadjuvant (chemo)radiotherapy, although considerably lower than after ICI. Our novel findings help elucidate real-world standard treatment patterns and outcomes, and provide context for the ICI study. With the emergence of smaller biomarker-based subgroups and novel targeted therapies, RWD is gaining relevance to gain insight into treatment patterns and outcome in clinical practice. To make optimal use of RWD, it is important that electronic health records contain high quality and complete data.

Biomarkers that are prognostic of recurrence after adjuvant CAPOX in CC

In addition to T and N stage and MMR status, we investigated novel biomarkers that are prognostic of recurrence after adjuvant CAPOX in CC. These biomarkers include circulating tumor DNA (ctDNA) (chapter 4) and the tumor-stroma ratio (chapter 5).

Based on international RCTs and observational studies, ctDNA is widely recognized as marker of minimal residual disease (MRD) after surgery, identifying patients who are not cured by surgery and need adjuvant treatment. In chapter 4, the observational PLCRC-PROVENC3 study, we confirmed that ctDNA-positivity post-surgery is a strong prognostic biomarker. The post-surgery ctDNA detection rate, absolute recurrence risk (RR), and hazard ratio (HR) were comparable to other stage III CC cohorts (Table 1). Moreover, compared to the patient-specific mutation panels designed in bespoke assays[24-27], our novel whole-genome sequencing (WGS) approach yields faster ctDNA results. This is important to timely inform treatment decisions, as ACT should be initiated preferably within eight weeks and no later than 12 weeks after surgery.[28-30] The presented WGS MRD assay is already implemented in a Dutch intervention study, to escalate from the guideline-based surgery only to ctDNA-based adjuvant CAPOX in low-risk stage II CC.[31,32] Patients who provide broad consent in PLCRC are randomized, only those in the intervention arm are approached for additional consent to disclose ctDNA status and if positive start ACT. Patients in the control arm receive standard surgery only. They are blinded to the ctDNA result, to prevent that ctDNA-

positive patients who are informed about their high RR would crossover from the surgery arm to the ACT arm. This trial within cohorts (TWiCs) design provides another alternative when a RCT is impractical.

In high-risk stage II and stage III CC, where ACT is already standard of care, the first aim would be to use ctDNA-negativity to identify patients who are cured by surgery alone and not in need of ACT. To safely de-escalate treatment, the negative predictive value of post-surgery ctDNA is currently insufficient. A considerable proportion of ctDNA-negative patients still experienced recurrence in chapter 4 and other studies (Table 1). Of the recurrences missed by post-surgery ctDNA, some reached the limit of detection in a later blood draw, underscoring the importance of ctDNA surveillance. In some patients with sequential false negative results, ctDNA was also undetectable pre-surgery. Pre-surgery, when the bulk tumor was still in situ, ctDNA was negative in 9% of patients in PROVENC3, compared to 16% in other studies[33,34]. In ongoing analyses on the PROVENC3 data, such unreliable negative or low ctDNA levels pre-surgery seem associated with smaller tumor diameter, less necrosis, less angioinvasion and distinct DNA and RNA features.[35,36] This data may help characterize patients who are prone to false negative ctDNA results and thus less suited for ctDNA-guided de-escalation. In addition, the post-surgery ctDNA result was more likely to be false negative in metastases to the peritoneum or lung.[34,37] This has been attributed to poor vascularization so lower ctDNA shedding, and the possibility that the peritoneum may be reached by local tumor growth rather than hematogenous spread.[38]

Because current ctDNA assays miss part of the recurrences, we investigated and confirmed the complementary value of tissue-based biomarkers like T and N stage (chapter 4) and stroma (chapter 5). T stage is determined on standard diagnostic slides, which can also be used to determine the tumor-stroma ratio (TSR). The UNITED study showed that patients with stroma-high CC (39%) had a worse 3-year event rate compared to stroma-low CC (34% versus 20%, HR 1.7).[45] We observed comparable prognostic impact of the TSR in chapter 5, supporting the robustness of the biomarker. Moreover, within the large subgroup of ctDNA-negative patients, we found that the RR after ACT (17%) was lower in patients with stroma-low tumors (9%) or pT1-3N1 stage (7%). The combination of ctDNA-negativity, stroma-low and pT1-3N1 identified a large subgroup of patients (34%) at very low-risk of recurrence (3%) who may currently be

overtreated with ACT. Whereas the TSR was manually determined by a trained pathologist and may encounter subjectivity, artificial intelligence (AI) algorithms can automatically classify stroma[46] or integrate all prognostic features on a slide.[47,48] Such digital pathology biomarkers may be less subjective and more efficient to implement in routine diagnostics.[49] Preliminary results of AI-based refined risk stratifications are promising in our ongoing analyses on patients with and without ACT.[50]

Table 1: Overview of circulating tumor DNA studies in stage III colon cancer, indicating % receiving ACT, % ctDNA-positive, hazard ratio and event rate per ctDNA status.
ACT, adjuvant chemotherapy; ctDNA, circulating tumor DNA; ddPCR, droplet digital polymerase chain reaction; NR, not reported, pt, patient; vs, versus; WGS, whole genome sequencing.

Study	Stage, treatment	ctDNA assay	Post-surgery ctDNA+/tested	Hazard ratio	Event rate ctDNA+ vs -
Chapter 4 PROVENC3	Stage III, 100% ACT	WGS (fixed)	28/209 (13%)	6.2 [3.4-11.2]	3-year 64% vs 17%
Tie[26]	Stage III, 99% ACT	Safe-SeqS (pt-specific panel)	15/88 (17%)	3.8 [2.4-21.0]	3-year 53% vs 24%
Kotani[24]	Stage III, 41% ACT	Signatera (pt-specific panel)	91/397 (23%)	9.6 [5.8-16.0]	1.5-year 51% vs 8%
Henriksen[25]	Stage III, 87% ACT	Signatera (pt-specific panel)	20/140 (14%)	7.0 [3.7–13.5]	3-year 80% vs 20%
Shah[39]	Stage III, 91% ACT	Signatera (pt-specific panel)	126/505 (25%)	10 [NR]	2-year 59% vs 8%
Li[40]	Stage III, 100% ACT	179 genes (fixed panel)	20/124 (16%)	3.6 [1.8-7.0]	3-year 54% vs 23%
Reinert[27]	Stage I-III, 62% ACT	Signatera (pt-specific panel)	10/94 (11%)	7.2 [2.7-19.0]	3-year 70% vs 12%
Chen[41]	Stage II/III, 73% ACT	GeneseeqPrime (fixed panel)	20/240 (11%)	11 [5.3-23]	2-year 61% vs 11%
Taieb[42]	Stage III, 95% ACT	Signatera (pt-specific panel)	109/564 (20%)	5.8 [4.2-7.9]	2-year 54% vs 12%
Sinicrope[43]	Stage III, 100% ACT	Guardant Reveal (tissue-free)	461/2260 (20%)	4.3 [3.7-5.1]	3-year 64% vs 17%
Benaim[44]	Stage II/III, 52% ACT	ddPCR (fixed panel)	18/171 (11%)	3.2 [1.3-7.9]	3-year 44% vs 14%

Biomarkers to predict response to treatment in CC

In addition to validating prognostic value of known clinicopathological biomarkers, we explored novel genomic and transcriptomic biomarkers that predict resistance to ACT. To isolate the response to adjuvant CAPOX from potential curation by resection, we used ctDNA-based MRD in chapter 6 and functional organoid models in chapter 7.

Consistent with the higher RR in stroma-high tumors, chapter 6 showed that gene expression signatures corresponding to mesenchymal consensus molecular subtype 4 (CMS4), epithelial mesenchymal transition (EMT) and cancer-associated fibroblasts (CAFs) were prognostic of recurrence after resection and ACT in pMMR/MSS CC.

However, only the CAF signature was also predictive of response to ACT in the subgroup of patients who have ctDNA-based MRD and rely on chemosensitivity to prevent recurrence. On genomic level, prognostic and predictive value was also observed for *SMAD4* mutation, a mediator in the TGF-β signaling pathway between CAFs and tumor cells. TGF-β signaling enhances tumor progression by reinforcing CMS4 traits and remodeling the extracellular matrix.[51] *SMAD4* mutation and CAF signatures had complementary prognostic value in chapter 6, as do *SMAD4* downregulation and TSR in prior studies.[52] TSR has also previously been shown to correlate with CMS4[53] and in some studies with FAP expression[54,55], which is a marker of CAFs that contributes to invasive tumor behavior through proteolytic activity[56] but was not prognostic in our chapter. In addition to above functions, the stroma may limit drug access by creating a physical barrier and by enzymatic degradation.[57,58]

The poor prognostic mesenchymal phenotype may provide leads for alternative treatment targeting CAFs and signaling pathways between stromal and tumor cells.[58] In patients with mCRC, intrinsic resistance to fluoropyrimidines has been associated with *SMAD4* mutations and has been restored by adding GB201.[59] Also the TGF-β inhibitor galunisertib has shown to improve pathological response to chemoradiation in RC.[60] FAP constitutes a target of the fibroblast activation protein inhibitor (FAPI, coupled with a radioisotope label for PET/CT scanning[61]), antibodies (sibrotuzumab, all progressive disease in mCRC[62]) and small molecules (talabostat, some stable disease in mCRC[63]). Pre-clinical work is ongoing on car-T cells[64] and bifunctional fusion proteins[65] targeting simultaneously FAP/TGF-β and PD-L1. CAFs may induce PD-L1 and thereby cause immune evasion and resistance to ICI[66,67], further highlighting the relevance of the tumor microenvironment (TME) in treatment response.

So far, trials with CAF-targeting therapies have shown limited efficacy[68], and various reasons have been proposed. First, most trials are performed in mCRC and we are not sure whether the stromal cells found in our study's (non-metastatic) primary tumors migrate along with the tumor cells in the formation of metastases. The TSR of the primary tumor does not correlate with the TSR of lymph node metastases[54], although high stroma is normally not found in lymph nodes and does add poor prognostic value[69]. It is plausible that TME–induced biomarkers are less stable during metastatic progression than tumor-intrinsic features and thus more difficult to target with systemic

treatment directed at (micro)metastases. Second, there are various CAF subtypes, including some with potential tumor-restraining functions.[70] Spatial transcriptomic analyses are ongoing on this cohort to obtain information on CAF subtypes[71], and localization relative to tumor cells and immune cells[72]. Second, The bulk RNA sequencing in chapter 6 does not provide information on whether the CAF genes are expressed solely by stromal cells or partly by tumor cells. The fact that the predictive value was not captured by currently known stromal markers, like TSR and FAP, may imply a tumor-intrinsic component. To move from TME-related to purely tumor-intrinsic biomarkers and treatment targets, isolated tumor cells can be cultured *in vitro*.

The aim of chapter 7 was to use patient-derived organoids (PDOs) as functional model to investigate tumor-intrinsic resistance.[73,74] Therefore, we first confirmed that PDO drug screens can predict response to CAPOX, consistent with other studies correlating area under the drug screen curve with event-free survival (HR 3.0 [1.4-6.4][75]; Spearman R=0.86[76]). Consistent with the 64% RR in ctDNA-based MRD, we defined two-thirds of the PDOs as resistant, including all the PDOs derived from patients with recurrence. Next, we compared CAPOX-resistant and CAPOX-sensitive PDOs to identify predictive biomarkers. Chemoresistance was characterized by downregulated proliferative signatures and upregulated EMT and TGF-β signaling, both consistent with the results in chapter 6. Our purely epithelial PDOs did not contain TME, but culturing in collagen or co-culturing with CAFs could induce mesenchymal gene expression.[77] Future studies should focus on co-cultures to further assess the impact of the TME on chemoresistance and to improve CAF-targeted treatment.[78,79]

Beyond the mesenchymal phenotype, it has proven difficult to find tumor-intrinsic gene expression profiles to direct personalized treatment. On genomic level, the rate of actionable mutations was low in chapter 6 and 7, as well as in prior publications.[34,74] On transcriptomic level, overexpression of *TACSTD2* in resistant PDOs may translate to protein expression of the targetable TROP2.[80] Proteomics may be better suitable to identify more treatment targets, as is currently investigated on these CC PDOs.[81] In addition to using PDOs to study (biomarkers for) resistance, PDOs can be derived from each individual patient to perform high-throughput drug screens to predict treatment response and inform treatment decisions.[82,83] However, this approach would be more cost- and time-consuming and may be more appropriate for refractory disease.

Future directions to improve personalized treatment in CC

Building on the findings and experience in this thesis, we propose future steps to combine biomarkers into a multimodal assessment of the tumor, and to integrate them into the clinical workflow. We outline the validation steps required for demonstrating predictive value of biomarkers for benefit from treatment, with the goal of enabling personalized therapeutic strategies. On the one hand, this includes identifying the ~50% of CC patients who are likely cured by surgery and may be considered for de-escalation of adjuvant treatment. On the other hand, we aim to pinpoint the ~30% of patients who remain at high risk of recurrence despite standard resection and adjuvant CAPOX, and to generate leads for escalation strategies based on TSR, molecular markers or PDOs (Figure 1).

Integration of biomarkers into combinations and into clinical practice

We presented several biomarkers with prognostic and even predictive value, but none demonstrated sufficient discriminative accuracy on their own. This highlights the need for a combined biomarker approach, to achieve clinically meaningful risk stratification and offer a multidimensional assessment of the tumor.[84] The current definition of high-risk stage II and III CC is based on T/N stage defining locoregional extent, and MMR status defining mutational burden and ICI response (chapter 3). The strongest novel biomarker is ctDNA, providing an indication of micrometastatic disease (chapter 4). In addition to tumor-intrinsic features like proliferation rate (chapter 6-7), components of the TME like low tumor-infiltrating immune cells (chapter 6) and high mesenchymal features (chapter 5-7) confer poor prognosis. The CAF signature has the strongest predictive value (chapter 6), but the microscopic tumor-stroma ratio (chapter 5) may be easier to implement in the current workflow. A tissue-based biomarker like TSR or AI algorithms can be applied on the resection tissue that is standardly used to determine pathological TN stage and MMR status. This could yield a quick result within a few days after surgery and help identify a low-risk group (pT1-3N1 stroma-low) for whom ctDNA-negativity helps select the very low-risk patients. Among the tissue-based high-risk group (pT4/N2 and stroma-high), the RR may be high irrespective of the more expensive ctDNA testing. Health technology assessment and cost-effectiveness analysis[85] should inform the optimal combination and implementation of the biomarkers, to identify patients eligible for de-escalation and escalation studies.[49]

Future directions for adjuvant de-escalation studies

This combination of biomarkers (ctDNA-negative, pT1-3N1, stroma-low) may help identify the very low-risk patients (3-year RR 3% and 5-year OS 95% after ACT). However, we are not sure whether these patients remain recurrence-free thanks to surgery (~50%) or thanks to ACT (~20%) and thus whether it would be safe to withhold ACT. To determine whether the low-risk patients derive >5% OS benefit from ACT, which is the minimum clinical benefit to approve adjuvant treatment[86,87], we envision the complementary use of RWD studies and RCTs. Our next real-world study determines the prognostic impact of the biomarker combination (ctDNA, T and N stage, TSR) on a larger observational cohort of high-risk stage II and III CC with and without ACT (ESMO 2025). In daily clinical practice, patients who forgo ACT are usually less fit (older, more comorbidity) and/or have less advanced tumors (lower T/N stage) compared to patients who receive ACT. To account for such confounders, the real-world treatment groups with and without ACT can be weighted or matched.[88] However, this only accounts for variables that are measured, with considerable residual confounding compared to RCTs.[16,17] Hence, the real-world results should help inform the design of a subsequent prospective trial. Ideally, we would randomize low-risk patients (ctDNA-negative, pT1-3N1, stroma-low) between three months adjuvant CAPOX and surgery only. To ensure compliance, a TWiCs design or biomarker-informed treatment decisions based on patient and doctor preference may be more suitable (Figure 1).

For the design of a biomarker-based de-escalation study, we can learn from international studies. In stage II CC, the DYNAMIC trial showed that ctDNA-based compared to guideline-based ACT resulted in more restrictive ACT use without inferior outcome.[89,90] In stage III CC, the observational GALAXY[24] and BESPOKE USA[91] contained some patients who did not receive the standardly recommended ACT. These studies suggested that the benefit of ACT is lower and not statistically significant in ctDNA-negative (HR 1.7 and 1.1, respectively) compared with ctDNA-positive patients (HR 6.6 and 3.9, respectively). Intervention trials are ongoing that use ctDNA-negativity in stage III CC to randomize between ACT conform the guideline and de-escalation to surgery only, including the DYNAMIC-III trial[92], CIRCULATE US[93] and the VEGA arm of CIRCULATE JAPAN[94] (Table 2). To reduce the risk of false negative results, some trials limit patient inclusion to only low-risk stage III (pT1-3N1) (NCT05529615) or only

patients who had detectable ctDNA pre-surgery (TRACC, also containing a standard of care arm not informed by ctDNA status[95]). In addition, as ctDNA may be missed in the first blood draw after surgery (chapter 4), most studies reassess ctDNA studies in serial blood draws to confirm that ctDNA remains undetectable. We would propose ctDNA surveillance to start systemic treatment once ctDNA becomes detectable.[96]

Future directions for adjuvant escalation studies

The other application of biomarkers is to identify patients at high risk of recurrence after standard CAPOX, and to inform an alternative effective treatment approach. The clinical need is demonstrated by the fact that adjuvant studies on alternatives to CAPOX have failed to improve outcome. As recently as 2025, tailored therapies like ICI in dMMR[97] and aspirin in case of PI3K alteration[98] have shown the first positive adjuvant results. Benefit was not demonstrated for i.a. irinotecan[99], bevacizumab[100], cetuximab[101] and celecoxib[102] (with the notable exception of the later MRD subgroup analysis[103]) (Table 2). One explanation for the limited benefit of adjuvant systemic therapy may be that approximately half of patients are cured by surgery alone, making recurrence-free survival not solely attributable to therapy sensitivity. In addition, the number of events is relatively low, and recurrence takes long to develop, resulting in large and lengthy trials. Trial efficiency may improve when using ctDNA to select patients who are not cured by surgery and rely on adjuvant treatment to prevent recurrence. This will be accompanied by a higher event rate and thus require a smaller sample size to maintain statistical power.[104] In addition, ctDNA may be used to monitor molecular relapses, preceding detection by standard imaging by months (7.6 months in chapter 4, 6-10 months in prior studies)[25,27]. Prospective studies should confirm whether earlier identification of patients at high RR helps inform treatment decisions that translate into a relevant benefit in OS. ctDNA clearance during ACT is associated with favorable prognosis (chapter 4)[24-26,40], but has also been observed spontaneously without systemic treatment[105,106]. Therefore, ctDNA clearance may not suffice as surrogate endpoint for response and establish outcome measures such as RR and OS will still be necessary.

Of the patients with ctDNA detection after surgery in chapter 4 and other studies (Table 1)[26,40], only one-third to half remain recurrence-free and/or clear the ctDNA after ACT. This indicates low chemosensitivity, especially in dMMR CC[43], and the need for other

personalized treatment. Studies are ongoing to investigate the ctDNA-based intensified radiological follow-up (IMPROVE-IT2) or ctDNA-informed treatment decisions by clinicians (BESPOKE). In the described trials for de-escalation in ctDNA-negative patients, ctDNA-positive patients are randomized between guideline-based treatment and escalation (Table 2). The first results of the DYNAMIC-III trial showed no improved curation of MRD patients with escalation from three months CAPOX to six months CAPOX or FOLFOXIRI.[92] This is in line with the similar efficacy of six and three months CAPOX in chapter 2 and the concurrent resistance of CAPOX-resistant PDOs to drug screens with irinotecan in chapter 7. In patients with ctDNA-positivity after CAPOX, trifluridine/tipiracil also provided no significant DFS improvement in preliminary results of the ALTAIR arm of CIRCULATE JAPAN (non-metastatic CRC)[107] and some benefit in INTERCEPT-TT (mostly mCRC)[106], with no cases of long-term cure in both cohorts. SU2C-ACT3 aimed to tailor treatment in patients who remained ctDNA-positive after standard ACT (only 14%), and randomized between surveillance and FOLFIRI, unless patient had an actionable biomarker (only 10%).[108] The SAGITTARIUS trial will randomize ctDNA-positive patients post-surgery per molecular subgroup (dMMR, *POLE, RAS/RAF*, *HER2*) between six months CAPOX/FOLFOX and targeted treatment.[96] Such platform trials are important to tailor therapy to an increasing variety of smaller molecular subgroups, either randomizing per molecular subgroup or comparing simultaneous single-arm intervention groups to a single control arm for more efficient inclusion.[109]

Future directions for neoadjuvant studies

This thesis described ctDNA and organoids to study isolated response to chemotherapy administered in the adjuvant setting. Another approach may be to move to the neoadjuvant setting, where pathological response can be directly quantified in the resection specimen. The FOxTROT trial showed lower RR when moving FOLFOX from the adjuvant to the neoadjuvant setting in pMMR CC but not in dMMR CC.[110,111] Benefit from chemotherapy was higher when adding panitumumab in *KRAS* WT CC[112] and other molecular targets are under investigation in the FOxTROT platform trial (Table 2)[113]. Pathological response correlated with RR, offering a more direct endpoint to provide additional insights into response to tailored treatment and predictive markers for response. The biomarkers in this thesis (ctDNA clearance[18], TSR[114] and PDO drug screens[76,115]) have been correlated with response to neoadjuvant treatment in RC. In

CC, neoadjuvant chemotherapy is only considered to improve resectability in cT4 pMMR.[116] The main hurdle for implementation of neoadjuvant treatment in clinical practice is the risk of overtreatment due to overstaging of especially clinical N stage on imaging, which may cause unnecessary toxicity and delay in surgical resection.[117,118] To select patients who are likely to benefit from neoadjuvant treatment, improved imaging modalities are needed. The role of pre-operative ctDNA is not yet consolidated, as it is not prognostic of RR or OS at baseline[119] but may prove of added value in monitoring response to neoadjuvant treatment[18].

Conclusion

RWD and blood- and tissue-based biomarkers have potential to improve prognostication and personalization of treatment in non-metastatic CRC. We show that shorter duration of adjuvant CAPOX can safely reduce overtreatment across T/N substages of CC. Post-surgery ctDNA-negative patients are at lower RR, especially if also pT1-3N1 and stroma-low. These patients may be considered for de-escalation to withhold ACT until ctDNA becomes detectable during surveillance. ctDNA-positivity allows for selection of patients not cured by surgery and in need of ACT. The majority of these patients appear resistant to the current standard CAPOX and are thus undertreated. Chemoresistance is characterized by high stroma, cancer-associated fibroblasts and epithelial mesenchymal transition. These mesenchymal markers, dMMR status and functional organoid models are promising to identify effective escalation strategies, as are molecularly targeted platform trials in the adjuvant MRD setting or neoadjuvant setting. With the emergence of therapies targeted at smaller molecular subgroups, traditional RCTs become challenging. Novel trial designs and high-quality RWD are gaining relevance to pave the way from one-size-fits-(not!)-all treatment to improved personalized treatment.

Figure 1: Proposed future studies: A) Post-surgery ctDNA-negative patients: de-escalation to ctDNA surveillance, starting ACT once ctDNA becomes detectable. B) ctDNA detection after surgery or after ACT: escalation from CAPOX to stroma-targeted, molecular-targeted (Table 2) or PDO-based treatment. In addition to recurrence, response can be monitored using ctDNA in the MRD adjuvant setting or pathological response in the neoadjuvant setting.
ACT, adjuvant chemotherapy; CAPOX, capecitabine and oxaliplatin; ctDNA, circulating tumor DNA; PDO, patient-derived organoid.

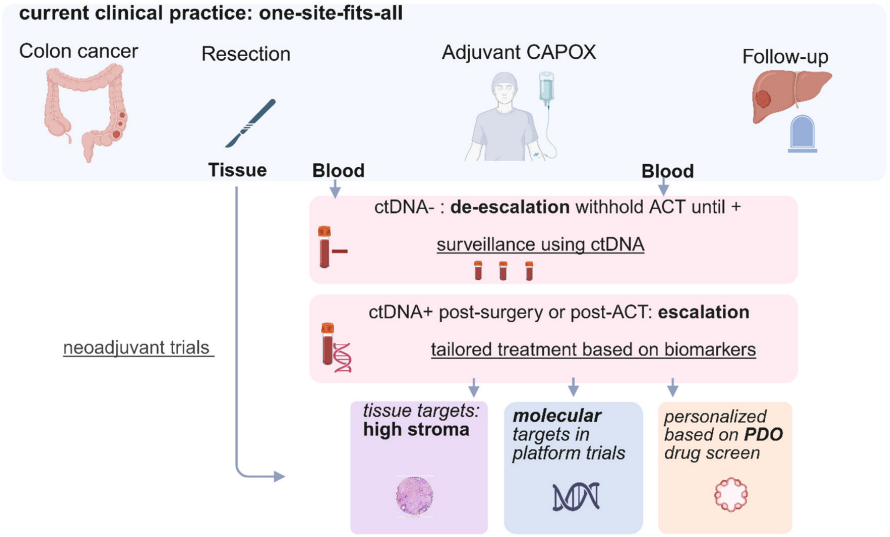


Table 2: Escalation trials in stage II-III colon cancer, depicted separately for A) neoadjuvant setting; B) adjuvant setting: irrespective of ctDNA testing, C) in ctDNA-based MRD post-surgery, D) in ctDNA-positivity after standard adjuvant CAPOX). E) X standard care in mCRC. *ACT, adjuvant chemotherapy; ctDNA, circulating tumor DNA; COX, cyclooxygenase; CTLA4, cytotoxic T-lymphocyte–associated protein 4; dMMR; deficient mismatch repair; EGFR, epidermal growth factor receptor; FTD/TPI, trifluridine/tipiracil (TAS-102); HER2, human epidermal growth factor receptor 2; PD(L)1, programmed death(ligand) 1; mCRC, metastatic colorectal cancer.*

Target	Treatment	A) Neoadjuvant Study	B) Adjuvant Study (no ctDNA test)	C) ctDNA+ post-surgery	D) ctDNA+ post-ACT	E) mCRC
-	CAPOX	CONNECTION[127]		DYNAMICIII[92]	NCT05529615	X
-	Irinotecan	FOxTROT3[113]	PETACC-3[99]	DYNAMICIII[92] ERASE-CRC[120]	PEGASUS[121]	X
-	FTD/TPI				ALTAIR[122] INTERCEPT-TT[106] ERASE-CRC[120]	X
COX2 -in PIK3CAmut	Celecoxib Aspirin		SWOG80702[102] ALASCCA[98]	>MRD subgroup[103]		
VEGF EGFR	Bevacizumab	NAVIGATE-CRC-01[128]	AVANT[100]			X
-in BRAF^{V600E}	Encorafenib+ Cetuximab	FOxTROT4[113]	A022004[123]		SU2C-ACT3[108]	X
-in RASwt	Panitumumab	FOxTROT1[110]		SAGITTARIUS[96]		X
-All/RASwt	Cetuximab	PRODIGE22[129]	PETACC-8[101]			
-RAF/RASwt	Cetuximab		Alliance N0147[43]	>MRD subgroup[43]		X
KRAS^{G12C}	Sotorasib/ Adagrasib					
HER2	Trastuzumab + Tucatinib / Pertuzumab			ERASE-CRC[120] SAGITTARIUS[96]		X
NTRK	Lacrotrectinib/ Entrectinib					X
dMMR -PDL1	Atezolizumab		ATOMIC[97]			
-PD1	Pembrolizumab	Neoprism[124] IMHOTEP[125]		NCT03832569		X
	Dostarlimab	FOxTROT5[110] AZUR2[126]				
+CTLA4	Ipilimumab + Nivolumab	NICHEII[21]		SAGITTARIUS[96]	SU2C-ACT3[108]	

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Appendices

-English summary
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English summary

In stage II/III colorectal cancer (CRC), treatment is still largely based on T and N staging and deficient or proficient mismatch repair status (dMMR or pMMR). Because a large proportion of patients are currently over- or undertreated with standard of care, this thesis aims to inform effective personalized treatment. The first part presents real-world data (RWD) to determine the impact of treatment guidelines and rare tumor subtypes in clinical practice. The second part investigates biomarkers that are prognostic of recurrence or predictive of response to adjuvant CAPOX in colon cancer.

Part I-Nationwide registry data to describe clinical practice in general population

In colon cancer (CC), adjuvant chemotherapy (ACT) with CAPOX is standard of care since 2004. In 2017, duration was reduced from six to three months, after randomized trials showed non-inferiority in high-risk stage II (pT4N0 pMMR) and low-risk III (pT1-3N1). Using RWD from the Netherlands Cancer Registry (NCR), chapter 2 shows a quick implementation and no difference in overall survival (OS) between patients receiving adjuvant CAPOX before (2015-2016, 5-year OS 81%) and after (2018-2019, 5-year OS 80%; hazard ratio (HR) 1.02) the guideline change. This remains the case in subgroup analysis of patients with high-risk stage III CC (pT4/N2; HR 1.06), for whom fear of undertreatment with three months persists internationally. Moreover, patients receiving three months CAPOX report less chemotherapy-induced peripheral neuropathy, a better quality of life, and a non-significant but clinically relevant improvement in ability to work, as late as two years after treatment. This study provides insights on the impact of trial results and guideline changes on daily clinical practice and supports the implementation of three months adjuvant CAPOX across all patients.

Nationwide patient registries like the NCR also help to characterize smaller subgroups of CRC with specific characteristics. In chapter 3, we compare all dMMR (2.3%) and pMMR stage II/III rectal cancer (RC) patients on patient and tumor characteristics, treatment patterns and outcome. Patients with dMMR RC are younger and have more advanced stages, poorly differentiated tumors, mucinous and signet ring cell histology, and *BRAF*^{V600E} mutations. In the total cohort, OS is better in dMMR than pMMR RC (HR 0.62). After matching dMMR to pMMR patients on i.a. age and stage, dMMR remains associated with better OS (HR 0.54), event-free survival (HR 0.43) and clinical and pathological complete response. These RWD findings help characterize dMMR RC and outcome after standard of care, to understand and further optimize treatment.

Part II – Biomarkers for recurrence risk and response to adjuvant CAPOX in CC

In chapter 4-6, we use the prospective Dutch Colorectal Cancer (PLCRC) cohort to collect tissue and blood for biomarker research for the PLCRC-substudy PROVENC3. In chapter 4, we describe a novel circulating tumor DNA (ctDNA) assay that is informed by whole genome sequencing on the resected tumor. Among patients with stage III CC, we show that recurrence risk (RR) is higher in patients with compared to without ctDNA detection after surgery (3-year RR 64% compared to 17%; HR 6.2) and after ACT (3-year RR 63% compared to 12%; HR 7.9). Post-surgery ctDNA testing has added prognostic value to pT stage, pN stage and MMR status in a multivariable Cox model. Part of the recurrences missed by post-surgery ctDNA testing are detected in serial follow-up and/or identified as high-risk stage III (pT4/N2). The combination of ctDNA with pTN stage improves risk stratification (3-year RR 82% for pT4/N2 ctDNA-positive compared to 7% for pT1-3N1 ctDNA-negative; HR 28.4).

Although ctDNA has high positive predictive value for recurrences, negative predictive value is insufficient. ctDNA fails to detect a proportion of recurrences, especially to the lung and peritoneum. Chapter 5 investigates the added value of the tumor-stroma ratio (TSR), which can be applied on the same H&E slide that is used for pTN staging. We show that 43% of all PROVENC3 patients have a stroma-high tumor, with increased 3-year RR compared to patients with a stroma-low tumor (33% compared to 16%; HR 2.7). Of the recurrences missed by ctDNA detection, 75% comes from a stroma-high primary tumor. The TSR thus adds prognostic value to ctDNA, as well as to pT4/N2 in a multivariable Cox model. A subgroup of ctDNA-negative patients with nevertheless high RR (40%, HR 14) is identified based on pT4/N2 and stroma-high. Conversely, a third of patients are ctDNA-negative and pT1-3N1 and stroma-low, they are at very low RR (3%) after ACT. These low-risk patients may be eligible for de-escalation of treatment.

The ctDNA-positive patients have minimal residual disease (MRD) after surgery and depend on sensitivity to adjuvant treatment for cure. In chapter 6, we compare ctDNA-positive patients with recurrence (CAPOX-resistant, 74%) and without recurrence (CAPOX-sensitive) to evaluate the predictive value of genomic and transcriptomic biomarkers for response to adjuvant CAPOX. In pMMR MRD, a high RNA sequencing signature indicative of cancer-associated fibroblasts (CAFs) has strongest predictive value for resistance to adjuvant CAPOX (HR 5.3). Predictive value is also observed for mutations in *SMAD4* (HR 3.4), a gene involved in TGF-β signaling between CAFs and tumor cells. The CAF signature and *SMAD4* mutation also have prognostic value for recurrence (34%) in the overarching pMMR cohort, independent of i.a. pTN stage. Other signatures, like consensus molecular subtype 4 and epithelial mesenchymal transition (EMT), are prognostic of recurrence in pMMR but not predictive of response in MRD. In the subgroup with dMMR CC (18%), less recurrences are observed (14%) and only pTN stage (HR 9.8) and CAF signature (HR 4.2) are prognostic. These biomarkers are consistent with a mesenchymal tumor phenotype in chemoresistance.

To determine whether we can model the patient resistance to adjuvant CAPOX *in vitro,* chapter 7 uses organoids derived from the resected high-risk stage II or stage III CC. The five patients with recurrence after adjuvant CAPOX have patient-derived organoids (PDOs) that are more resistant than the five patients without recurrence, thus providing a good model to study tumor-intrinsic resistance in treatment-naïve tumor cells. The CAPOX-resistant PDOs are in most cases also resistant to the first-line alternative irinotecan and show limited genomic mutations to inform alternative drug screens. On transcriptomic level, resistance is characterized by upregulated TGF-β signaling and genes involved in EMT, as well as actionable genes like *TACSTD2* encoding the targetable protein TROP2. The identified biomarkers are validated in the resection tissue of a larger clinical cohort, namely the one described in chapter 6.

Together, these chapters show the potential of real-world data and of biomarkers to identify which patients are over- and undertreated in current clinical practice. Prognostic and predictive biomarkers have the potential to shift the current one-size-fits-all standard of care toward personalized treatment of non-metastatic CRC.

Dutch summary

(Nederlandse samenvatting)

Huidige standaardbehandeling

Colorectaal carcinoom (CRC) is een veelvoorkomende vorm van kanker, en wereldwijd de op één na belangrijkste oorzaak van kanker gerelateerde sterfgevallen. In 2023 kregen in Nederland circa 9.000 mensen de diagnose dikkedarmkanker (coloncarcinoom; CC) en 3.000 de diagnose endeldarmkanker (rectumcarcinoom; RC). Behandeladviezen worden momenteel gebaseerd op tumor doorgroei (T stadium), lymfeklier betrokkenheid (N stadium) en de aanwezigheid van uitzaaiingen (afstandsmetastasen; M stadium). Daarnaast wordt er onderscheid gemaakt tussen de aan- of afwezigheid van de “mismatch repair genen” (MMR) die een rol spelen in herstel van DNA. In niet-gemetastaseerd CRC (M0), is operatieve verwijdering (resectie) van de primaire tumor de hoeksteen van de behandeling. In CC wordt na de resectie aanvullend (adjuvant) chemotherapie geadviseerd in geval van stadium III (pN1 of pN2) of hoog-risico stadium II (pT4N0 met proficient mismatch repair (pMMR)).

Slechts bij 20% van patiënten met niet-gemetastaseerd CC wordt terugkeer van ziekte (recidief) voorkomen dankzij deze adjuvante chemotherapie (ACT). De helft is al genezen door de resectie en heeft dus geen ACT nodig. Deze overbehandeling zou hen bespaard kunnen worden, als er biomarkers worden gevonden om deze laagrisico patiënten vooraf te identificeren. Nog eens 30% krijgt recidief ziekte en is dus niet genezen door de resectie en niet door de ACT, zij worden ontoereikend behandeld met de huidige standaardzorg. Biomarkers zijn nodig om deze hoog-risico patiënten te identificeren en om aanknopingspunten te vinden voor welke behandeling wel zou werken. Sinds 2004 bestaat standaard-ACT uit CAPOX (capecitabine en oxaliplatine). Deze one-size-fits-all helpt niet elke patiënt, daarom is betere behandeling op maat nodig. Gerichte behandelingen die aangrijpen op deficiënte mismatch repair status (dMMR; minder gevoelig voor chemotherapie, meer gevoelig voor immuuntherapie) of DNA-mutaties (*BRAF/KRAS*; gevoelig voor doelgerichte therapie) zijn vooralsnog niet opgenomen in de richtlijn voor niet-gemetastaseerd CRC.

Het doel van dit proefschrift is om bij te dragen aan betere gepersonaliseerde zorg voor patiënten met niet-gemetastaseerd colorectaal carcinoom – door middel van het onderzoek op basis van van real-world data (RWD) (**deel I)** inclusief subgroepen op basis van stadium en MMR status, en biomarkers (**deel II**) zoals circulerend tumor DNA en tumor-stroma ratio en organoïden.

Deel I Real-world data over standaardbehandeling in de algehele CRC populatie
RWD omvat informatie uit dossiers van patiënten in de dagelijkse praktijk en geeft een meer representatief beeld van de algemene populatie dan data die afkomstig zijn uit klinische studies. Klinische studies includeren namelijk vaak patiënten die jonger en fitter zijn, en hanteren strikte behandelvoorschriften en controles die in de dagelijkse praktijk niet altijd haalbaar zijn. Dit kan resulteren in studieresultaten die niet representatief zijn voor de 'real-world' situatie. Daarom geeft RWD noodzakelijke inzichten in de toegevoegde waarde van behandelresultaten in de dagelijkse praktijk, inclusief relevante subgroepen op basis van eigenschappen van de patiënt (zoals leeftijd) of de tumor (zoals dMMR status). Dit proefschrift gebruikt twee RWD-bronnen: de Nederlandse Kankerregistratie (NKR) bevat landelijke gegevens. Patiënten kunnen daarnaast binnen het Prospectieve Landelijke CRC (PLCRC) cohort vragenlijsten invullen en toestemming geven voor gebruik van bloed en weefsel voor onderzoek.

De eerste toepassing van RWD in dit proefschrift is de beoordeling van de impact van een richtlijnwijziging op de dagelijkse praktijk. In 2017 is de behandelduur van adjuvant CAPOX in CC aangepast van zes naar drie maanden, nadat de internationale IDEA studies vermindering van toxiciteit aantoonden zonder klinisch relevante verslechtering in ziektevrije overleving. Deze non-inferioriteit voldeed niet aan de statistische voorwaarden voor de subgroep met hoog-risico stadium III CC (pT4/N2), daarom adviseren internationale richtlijnen vaak nog een behandelduur van zes maanden voor deze groep. Nederland is uniek in het adviseren van drie maanden CAPOX voor alle patiënten met hoog-risico stadium II en stadium III CC.

Hoofdstuk 2 richt zich op NKR-data van alle patiënten die in Nederland CAPOX krijgen, om een behandelduur van zes maanden CAPOX voor de richtlijnwijziging (2015-2016) te vergelijken met een behandelduur van drie maanden CAPOX na de richtlijnwijziging (2018-2019). De resultaten bevestigen een vergelijkbare 5-jaarsoverleving (~80%), onafhankelijk van o.a. leeftijd en MMR status, óok in hoog-risico stadium III (pT4/N2). Daarnaast tonen PLCRC-vragenlijsten minder lange termijn bijwerkingen na drie maanden CAPOX: minder zenuwschade, betere kwaliteit van leven en een beter werkvermogen. Samengenomen bevestigt dit hoofdstuk dat de studie-gebaseerde richtlijnverandering van zes naar drie maanden CAPOX veilig doorgevoerd kan worden in de praktijk, en helpt met inperken van overbehandeling en bijwerkingen.

Daarnaast kan RWD ook ingezet worden om behandelpatronen en uitkomsten in kaart te brengen van patiënten met een zeldzame tumorsoort. Een voorbeeld is dMMR, wat minder vaak voorkomt (2-5%) en slechter gekarakteriseerd is in RC dan in CC. Een recente klinische studie toonde dat neoadjuvant immuuntherapie (dostarlimab voor de operatie) een complete klinische respons gaf in alle 59 patiënten met dMMR RC, waardoor deze patiënten een operatie en bijbehorende complicaties zoals een stoma bespaard konden blijven. Dit was een single-arm studie met alleen immuuntherapie, en bevatte geen controlegroep om een vergelijking te kunnen maken met de huidige standaardzorg (neoadjuvante (chemo)radiotherapie). Hoofdstuk 3 beschrijft op basis van NKR-data het grootste cohort tot op heden van stadium II/III dMMR RC en vergelijkt dit met pMMR RC. dMMR RC (2.3%) is geassocieerd met jongere leeftijd, hoger stadium, slechte differentiatie, meer mucineus of zegelringcellen, en vaker een mutatie in het *BRAF*-gen. dMMR patiënten tonen een betere overleving dan pMMR patiënten, ook als alleen pMMR patiënten met vergelijkbare kenmerken beschouwd worden (gematcht met dMMR op o.a. leeftijd en stadium). Tevens tonen dMMR patiënten vaker een complete respons op neoadjuvante behandeling, op basis van pathologische beoordeling van het weefsel of klinisch met scans (30% versus 12%), wat betekent dat operatie mogelijk niet meer nodig is. Deze real-world resultaten geven inzichten in respons van dMMR RC op standaardzorg, en helpen met de interpretatie van de respons op immuuntherapie in single-arm studies.

Deel II Biomarkers voor stratificatie van recidief risico na adjuvant CAPOX in CC

Om beter te bepalen wie baat heeft van welke behandeling, zijn meer biomarkers nodig dan pathologisch T/N-stadium en MMR status. Biomarkers zijn meetbare kenmerken van de patiënt (zoals leeftijd en geslacht) of de tumor (pathologisch of moleculair) met prognostische waarde voor recidief ziekte of overlijden, en/of met predictieve waarde voor gevoeligheid of resistentie voor CAPOX en alternatieve behandelingen.

Prognostische biomarkers: circulerend tumor DNA en tumor-stroma ratio

Naast stadium kan een patholoog andere hoog-risico kenmerken scoren in het tumorweefsel, zowel van de tumorcellen (o.a. histologie en slechte differentiatie) als van de omliggende cellen (o.a. veel steuncellen (fibroblasten of “stroma”) of weinig immuun cellen). Daarnaast kan bloed onderzocht worden op de aanwezigheid van

circulerend tumor DNA (ctDNA) dat is afgegeven door tumorcellen. Na operatie is detectie van ctDNA een aanwijzing dat niet alle tumorcellen verwijderd zijn. Er is dan sprake van minimale restziekte (MRD), in de vorm van kleine uitzaaiingen (micro-metastasen) die nog niet te zien zijn op scans. Deze kunnen uitgroeien tot een recidief als ACT niet aanslaat. ctDNA is een veelbelovende biomarker om gerichter te selecteren wie niet genezen is door operatie alleen (ctDNA-positief) en dus ACT nodig heeft, en wie op de ACT reageert (ctDNA klaart) of niet (ctDNA-positief blijft of wordt).

Hoofdstuk 4 onderzoekt de prognostische waarde van postoperatief ctDNA in patiënten met stadium III CC, die volgens de richtlijn behandeld zijn met adjuvant CAPOX. ctDNA wordt gedetecteerd bij 13% van de patiënten (eerdere studies ~15%) en heeft toegevoegde waarde op pT en pN-stadium en MMR-status in het voorspellen van recidief, 7.6 maanden voordat metastasen aantoonbaar zijn op een scan (eerdere studies 6-10 maanden). De ctDNA-positieve patiënten hebben een 3-jaar recidief risico (RR) van 64% (eerdere studies 40-80%). Dit betekent dat twee-derde van de patiënten met MRD niet is genezen door adjuvant CAPOX, en dus slechts een-derde gevoelig is. ctDNA-negatieve patiënten hebben een lager RR, namelijk 17% (eerdere studies 8-24%). Dit toont dat een aanzienlijk deel van de ctDNA-negatieve patiënten toch recidief ziekte ontwikkelt, bij hen wordt de MRD gemist door de huidige ctDNA test.

Een deel van de door ctDNA gemiste recidieven is wel te voorspellen met weefsel biomarkers: hoofdstuk 4 toont dat RR nog maar 7% is in de ctDNA-negatieve patiënten met laag-risico stadium III (geen pT4/N2) en hoofdstuk 5 dat dit nog slechts 3% is in de subgroep die daarnaast een tumor heeft met laag stroma (≤50%). De tumor-stroma ratio (TSR) kan gescoord worden op dezelfde standaard weefselcoupe als het stadium, volgens instructies in de UNITED e-learning. Vergeleken met de UNITED studie, beschrijft hoofdstuk 5 een vergelijkbare proportie aan stroma-hoge tumoren (43%) en verhoogd RR (stroma-hoog 33% versus stroma-laag 16%). Van de ctDNA-negatieve patiënten met desondanks recidief, met name in de longen en buikvlies (peritoneum), heeft 75% een stroma-hoge tumor. Een-derde van alle patiënten is ctDNA-negatief met geen pT4/N2 en laag stroma, van hen ontwikkelt slechts 3% een recidief na 3 jaar en 5% overlijdt na 5 jaar. Vervolgstudies moeten aantonen of het veilig zou zijn om deze laag-risico patiënten ACT te besparen in de toekomst. De ctDNA-negatieve patiënten met desondanks een hoog recidief risico (40%) worden

gekenmerkt door pT4/N2 en hoog stroma. Zij zijn ongevoelig voor CAPOX en hebben een andere behandeling nodig, wat zich naar aanleiding van deze en andere studies zou kunnen richten op stroma.

Predictieve biomarkers: fibroblasten en organoïden

Patiënten zonder recidief ziekte kunnen genezen zijn door de resectie of door de ACT. Daarom is het lastig om van biomarkers de voorspellende waarde voor gevoeligheid voor ACT vast te stellen. Gen mutaties (DNA) in *BRAF* en *KRAS* zijn geassocieerd met slechtere uitkomst na chemotherapie, maar doelgerichte behandelingen die hierop aangrijpen hebben geen overlevingswinst gebracht in adjuvante studies van niet-gemetastaseerd CC. Genexpressie (RNA) maakt onderscheid tussen vier consensus molecular subtypes (CMS), waarvan CMS4 gekenmerkt wordt door veel stroma en fibroblasten en slechte prognose na chemotherapie. Om predictieve biomarkers (DNA en RNA) te bestuderen voor gevoeligheid voor CAPOX (geïsoleerd van genezing door operatie), beschrijft dit proefschrift postoperatief ctDNA om patiënten met MRD te selecteren en van organoïden om de tumor en de respons na te bootsen in het lab.

Hoofdstuk 6 richt zich op prognostische en predictieve biomarkers op basis van DNA- en RNA-sequencing, door stadium III CC patiënten met en zonder recidief na resectie en adjuvant CAPOX te vergelijken. In de pMMR subgroep worden patiënten met recidief gekenmerkt door pT4/N2 stadium, rechtszijdige tumoren en invasie in lymfe- en bloedvaten. Ook genexpressie passend bij CMS4 en epitheliale mesenchymale transitie (EMT) heeft prognostische waarde in pMMR. Een hoge genexpressie passend bij kanker-geassocieerde fibroblasten (CAFs) is de enige biomarker met prognostische waarde in pMMR (3-jaar RR 41% vergeleken met 18%) én in dMMR (27% vergeleken met 7%). Bovendien heeft alleen de CAF-expressie predictieve waarde voor respons op ACT in de subgroep met MRD, zij zijn niet genezen door de operatie en dus afhankelijk van gevoeligheid voor ACT. Van de pMMR MRD-patiënten zijn degenen met recidief (ondanks ineffectieve ACT) vaker CAF-high, en degenen die die recidiefvrij blijven (dankzij de chemotherapie) vaker CAF-low. Daarnaast tonen pMMR en MRD-patiënten met recidief meer mutaties in *SMAD4,* betrokken in communicatie tussen tumorcellen en stromale CAFs (o.a. via TGF-β). *SMAD4*-gerichte behandeling is nog in ontwikkeling.

Om te onderzoeken of CAPOX-resistente tumoren wél gevoelig zijn voor andere behandelingen, zijn tumor modellen nodig. Uit het operatieweefsel kunnen tumorcellen gekweekt worden tot een mini-tumor (organoïde). De organoïde lijkt op de tumor qua mutaties en gevoeligheid voor medicijnen: als een organoïde doorgroeit of doodgaat bij toevoegen van een medicijn (drug screen), geldt dit vaak ook voor de tumor. Dit is bekend van metastasen van CRC die groot genoeg zijn om te vervolgen op een scan, maar is lastiger te beoordelen voor micrometastasen na resectie van niet-gemetastaseerd CC. Hoofdstuk 7 toont dat organoïden van patiënten met recidief ziekte na adjuvant CAPOX resistenter zijn dan organoïden van patiënten zonder recidief. Deze resistentie wordt op basis van RNA-sequencing gekenmerkt door EMT en TGF-β signalering. Daarnaast tonen CAPOX-resistente organoïden hoge expressie van het gen *TACSTD2*, wat een eiwit codeert (TROP2) dat een mogelijk aangrijpingspunt vormt voor nieuwe medicijnen. De resistente organoïden tonen geen DNA-mutaties die aanleiding geven tot drug screens met bekende doelgerichte behandelingen. Slechts één CAPOX-resistente organoïde (en bijbehorende patiënt) blijkt gevoelig voor irinotecan, de eerste keuze bij metastasering. Dit benadrukt de noodzaak voor nieuwe behandelingen, in onderzoek hiernaar kunnen aan de organoïden fibroblasten toegevoegd worden.

Conclusie en adviezen op weg naar gepersonaliseerde behandeling

Samengenomen toont dit proefschrift dat patiënten met een hoog risico op recidief na standaardbehandeling gekenmerkt worden door pT4/N2 (grote tumor), pMMR (beter DNA-herstel, minder immuunreactie), ctDNA (restziekte na operatie) en hoog stroma (steunweefsel). Een combinatie van deze factoren (ctDNA-negatief, geen pT4/N2, stroma-laag) is nodig om patiënten met een laag recidief risico na adjuvant CAPOX te selecteren voor minder overbehandeling. RWD kan aantonen of dit risico ook laag is in een observationele controlegroep zonder ACT, en of de overlevingswinst dankzij ACT opweegt tegen het risico op bijwerkingen. Vervolgstudies gericht op minder overbehandeling (de-escalatie) kunnen deze laag-risico patiënten na operatie vervolgen met bloedafnames, om pas chemotherapie te starten als ctDNA detecteerbaar wordt. De combinatie van factoren helpt ook met identificeren van patiënten met hoog RR na adjuvant CAPOX, op basis van bloed (kleine groep ctDNA-positief) of weefsel (grotere groep pT4/N2 en stroma-hoog). Met verschillende technieken tonen we een rol voor mesenchymale kenmerken: stroma, *SMAD4*-mutatie, en genexpressie passend bij fibroblasten, EMT en TGF-β. Dit kan aanknopingspunten bieden voor gerichte behandelingen, die mogelijk wel effectief zijn in CAPOX-resistente patiënten die nu onderbehandeld worden (escalatie).

Voor nieuwe behandelingen kunnen we leren van stroma en organoïden. ctDNA kan helpen om adjuvante studies efficiënter maken, door patiënten te selecteren die na operatie nog behandeling nodig hebben (ctDNA-positief) en door respons (ctDNA klaring) en recidief (ctDNA terugkeer) te meten. Een andere optie is om behandelingen neoadjuvant te geven, om pathologische respons te meten. Zowel in de neoadjuvante context als in het geval van MRD na operatie, lopen er momenteel nieuwe platform studies waarin parallel verschillende moleculaire subgroepen doelgericht worden behandeld. Dergelijke innovatieve biomarker-gestuurde studieopzetten, en de inzet van real-world data, kunnen bijdragen aan de overgang van standaardzorg naar effectievere gepersonaliseerde behandeling van niet-gemetastaseerd CRC.

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List of publications

1. **Franken I.A.**, Smabers L.P. , Lunenberg R.A., Al Toma D., Roodhart J.M.L., Kwakman R.J.J.M., Intven M.P.W., Bol G.M., Koopman M. (2025) Onderste deel tractus digestivus: colorectaal carcinoom. *NED TIJDSCHR ONCOL*; 22:127-34.

2. **Franken I.A.**, van der Baan F.H., Vink G.R., May A.M., van Grevenstein W.M., Roodhart J.M., Koopman M. (2024) Survival and patient-reported outcomes of real-world high-risk stage II and stage III colon cancer patients after reduction of adjuvant CAPOX duration from 6 to 3 months. *European Journal of Cancer*; 208.

3. **Franken I.A.**, van der Baan F.H., Roodhart J.M., Koopman M., Vink G.R., May A. M., & van Grevenstein W.M. (2025) Reply to: Putting IDEA's Results Into Practice: Practicality Should Rule Complexity in Stage III Colon Cancer. *JCO Oncology Practice*; OP-25.

4. Lunenberg R.A.*, **Franken I.A.***, van der Baan F.H., Sijtsma F.P., Vink G.R., Braat M.N.G.J.A., Moons L., Lacle M.M., Elferink M.A.G., Boklage S., Intven M.P.W., Garside J., Koopman M., van Wuijtswinkel R., Roodhart, J. (2025) A comparison by mismatch repair status of real-world characteristics and outcomes in patients with stage II/III rectal cancer in the Netherlands. *Intl Journal of Cancer*.

5. Lunenberg R.A., **Franken I.A.**, Braat M.N.G.J.A., Elferink M., van der Baan F.H., Koopman M., Vink G.R., Intven M.P.W., Sijtsma F.P.C, Roodhart J.M.L. (2025) The impact of mismatch repair status on accuracy of clinical staging in stage II/III rectal cancer in daily practice. *European Jounrnal of Cancer;* Accepted for publication.

6. Rubio-Alarcón C., **Franken I.A.***, Andrew G.*, Wang H., van Nassau S.C.M.W., Schraa S.J., van der Kruijssen D.E.W., van Rooijen K., Linders T.C., Delis-van Diemen P., Alkemade M., Bolijn A., Tijssen M, Lemmens M, Meiqari L., Ketelaars S.L.C., Closa-Mosquera A., van Dongen M.M.W., Lanfermeijer M., Lissenberg-Witte B.I., Bosch L.J.W., Bisschop-Snetselaar T, Adriaans B.C., Greer A., Riley D., White J.R., Greco C., Cox L., Fox J., Victor K., Leech C., Angiuoli S.V., Kok N.F.M., Punt C.J.A., van den Broek D., Koopman M, Meijer G.A., Velculescu V.E., Roodhart J.M.L., Coupé V.M.H., Sausen M., Vink G.R., Fijneman R.J.A. (2025) ctDNA in patients with stage III colon cancer: the multicenter prospective PROVENC3 study. *British Journal of Surgery;* Accepted for publication (pending minor revisions).

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9. **Franken I.A.**, Ketelaars S.L.C., Rubio-Alarcón C., Delis-van Diemen P., Spaapen T., Heilijgers F., da Silva M., van Nassau S.C.M.W., Schraa S.J., Mesker W.E., de Leng W.J., Kranenburg O., Meijer G.A., Koopman M., Sausen M., Vink G.R., Abeln S., Fijneman R.J.A.*, Roodhart J.* (2025) Cancer-associated fibroblast signature and SMAD4 mutation in resistance to adjuvant chemotherapy in stage III colon cancer patients. *Under review.*

10. **Franken I.A.**, Smabers L.P., Huismans M.A., Brunner S., van Hoeck A., Beerling E., van Gestel E., Otten J.M.M.B., Koopman M., Fijneman R., Kranenburg O., Roodhart J.M.L. (2025) Patient-derived colon cancer organoids to model adjuvant chemoresistance and identify predictive biomarkers. *Under review.*

11. Huismans M.A.*, Smabers L.P.*, Brunner S.R., van Hoeck A., van der Kaa D., **Franken I.A.**, Wensink G.E., Koster J., Volckmann R., Kranenburg O., Koopman M., Snippert H.J.G, Roodhart J.M.L. (2025) Impact of treatment history on drug resistance of metastatic colorectal cancer organoids. *iScience*; 28(11): 113801.

12. **Franken I.A**., Busslinger G., Weusten B., Brosens L.A., Ruurda J.P., Haj Mohammad N., Mook S., Clevers H., Van Hillegersberg R. (2025) Patient-derived cancer organoids to tailor personalized treatment strategies in upper gastro-intestinal malignancies. *Diseases of the Esophagus*; Accepted for publication.

13. Busslinger G.A., Lissendorp F., **Franken I.A.**, van Hillegersberg R., Ruurda J.P., Clevers H., & de Maat M.F. (2020) The potential and challenges of patient-derived organoids in guiding the multimodality treatment of upper gastrointestinal malignancies. *Open Biology*; 10(4): 190274.

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16. Berbers R.M., **Franken I.A.**, & Leavis H.L. (2019) Immunoglobulin A and microbiota in primary immunodeficiency diseases. *Current opinion in allergy and clinical immunology*; 19(6): 563-570.

*Equal contribution

PhD portfolio

Conferences and symposia

Dutch cfDNA symposium	2022
ESMO molecular analysis for precision oncology	2022
ISMRC International symposium on minimal residual cancer	2023
AACR American association for cancer research	2024
ESMO European society for medical oncology	2024
ASCO American society of clinical oncology	2025

Other presentations or media appearances

SUMMA symposium: poster on MEDOCC-CrEATE	2022
PLCR anniversary: presentation on PROVENC3 for involved staff	2023
UMCU research day: poster on Green Team Research	2023
Oncomid publiekslezing: presentation for patients with CRC	2024
PLCRC publiekslezing: presentation for participants in PLCRC	2025
Oncologie.nu: ASCO25 - Nederlandse posters in de kijker CRC	2025
Oncologie.vandaag interview oncologie vandaag	2025

Teaching – qualification uitvoeren van onderwijs (UvO)

Interdisciplinary collaborations: medical students and nurses	2023
Clinical reasoning in internal medicine: SUMMA year 1	2023-2024
Clinical Scientific research: CRU year 3	2025
Supervising bachelor student Bobbie: literature review dMMR	2023
Supervising bachelor student Sophia: organoid experiments	2023-2024
Supervising master student Daan: in RNA seq data analysis	2023-2024

Grant and merits

Stichting Sacha Swarttouw-Hijmans: Tumor-T cell Location	€25.000
TKI-UMCU internal call: DoMore prognostic and predictive value	€675.000
HealthHolland International: kickstarter for public-private partnership	€303.600
AACR scholar-in training award 2024	€1800
ASCO merit award 2025	€1000
Travel grant Girard de Mielet van Coehorn 2024	€625

Other activities

Study coordinator of nationwide MEDOCC and MEDOCC-CrEATE	2022-2024
Screening of study participants for ia Xilis Princess Maxima Center	2022-2025
Overview chapter on CRC Nederlands Tijdschrift voor Oncologie	2025
Green Team Research: sustainability initiatives	2022-2024
MD PhD committee: representation and career events	2022-2025
Lustrum committee of the Clinical and Translational Oncology program	2022
Participant PhD Career Boost Talent Program, UMC Utrecht	2024-2025
Clinical work at emergency department and oncology ward	2022-2023

About the author

Ingrid grew up in Rotterdam and graduated from Emmauscollege in 2013. Next, she pursued her broad interest at University College in Utrecht, combining the pre-medical track with a minor in psychology and a semester abroad to the University of Leeds. In 2016, she enrolled in the Selective Utrecht Medical Master to become a medical doctor with senior internships in oncology and gastroenterology. Ingrid made her first strides in clinical practice in 2021, working in internal medicine at the Diakonessenhuis.

Ingrid got acquainted with upper gastrointestinal cancer organoids to personalize treatment as student in Clevers lab. This experience motivated a similar PhD project in the lower gastrointestinal tract, in the Lab of Translational Oncology under supervision of dr. Roodhart, dr. Fijneman and prof. Koopman. In addition, she collaborated with the Netherlands Cancer Institute to coordinate a nationwide intervention trial on circulating tumor DNA, as well as observational studies on DNA/RNA sequencing and tissue biomarkers to predict prognosis in colorectal cancer.

Beyond the scope of this thesis, Ingrid gained experience with other aspects of initiating and conducting research. She helped obtain funding and develop protocols for novel (public-private) partnerships on micro-organospheres, digital pathology biomarkers, spatial transcriptomics and proteomics. She had the opportunity to present her research at conferences and for patients (Oncomid, PLCRC). She also enjoyed mentoring multiple interns and teaching *(*University Teaching Qualification).

During her PhD, Ingrid participated in the PhD talent program to develop leadership and communication skills required in research. She was also devoted to representing fellows in the MD PhD committee and to raising awareness for sustainability in the Green Team Research. During her time as researcher, Ingrid followed her passion for patient care by working shifts at the emergency department and the oncology ward. She is looking forward to start her residency in Internal Medicine in Utrecht.

In her spare time, Ingrid also likes to challenge herself, whether it is to learn new crafts like photography or sports like (ice) skating, or to follow Senny on outdoor adventures – from high in the alps (skiing, climbing or hiking) to deep in the ocean (scuba diving).

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