Colon Cancer

-*MATCH*ing patients and treatment-

Inge van den Berg

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- MATCHing patients and treatment-

Colon Carcinoom

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Introduction

GENERAL INTRODUCTION

Colorectal cancer (CRC) is the third most common malignancy in men and the second most common in women with an incidence of 1.8 million worldwide. In the Netherlands, around 14,000 patients were newly diagnosed with CRC and nearly 5,000 patients died as a result in 2018 [1]. Based on the location of the tumor, CRC can be divided in two types of cancers, namely colon cancer and rectal cancer. Although both types of cancer have similarities in behavior, for which they are often taken together in literature and research, differences regarding staging, treatment and prognosis between the two exists. Most of the performed studies in this thesis include patients with colon cancer, therefore this introduction will mainly focus on this type.

Prognosis of patients diagnosed with colon cancer is dependent on multiple factors, with tumor stage being one of the most important. The International Union Against Cancer tumor node metastasis (TNM) classification is the globally recognized standard for staging malignant tumors [2]. This classification system consists of three categories: the T-category describes the size of the original primary tumor, the N-category describes lymph node involvement and the M-category describes the absence or presence of distant metastasis. Combined, these three categories (TNM-stage) can be described by stage I, II, III or IV (Table 1.) Survival for patients with non-metastatic disease (stage I-III) can be substantially different, depending on the T- and N-stage. Next to differences in tumor characteristics, several patient factors such as obesity, smoking and nutritional status have been investigated and associated with survival, yet much of the disparity in prognosis remains unexplained [3-6].

Table 1. TNM classification for colon cancer

	Tumor stage	T	N	M
	0	Tis	N0	M0
Low risk	I	T1 or T-2	N0	M0
	II	T3	N0	M0
High risk	II	T4	N0	M0
	III	Any T	N1-N2	M0
	IV	Any T	Any N	M1

Tis (Carcinoma in situ): Intraepithelial or intramucosal carcinoma (involvement of lamina propria with no extension through the muscularis mucosa); T1: Tumor invades submucosa (through the muscularis mucosa but not into the muscularis propria; T2: Tumor invades muscularis propria; T3: Tumor invades through the muscularis propria into the pericolorectal tissues; T4: Tumor invades the visceral peritoneum or invades adjacent organ or structure; N0: No regional lymph node metastasis; N1: Metastasis in 1-3 regional lymph nodes; N2: Metastasis in 4 or more lymph nodes; M0: No distant metastasis; M1: Metastasis to distant sites or organs

MATCH-study

To be able to identify better predictive and prognostic markers for colon cancer patients, the MATCH-study (MicroArray and proteomics Technologies to analyse Colorectal cancer and Hepatic metastasis) was initiated in 2007 [7-9]. In this prospective multicenter cohort registry, patients with stage I-III CRC who underwent surgery with curative intent in the large region of Rotterdam in the Netherlands, were enrolled between 2007 and 2017. All patients provided written informed consent for the collection of long-term clinical data and storage of tissue samples. Nearly 3000 CRC patients were included from eight participating centers (Erasmus Medical Center, Maasstad Hospital, Ikazia Hospital, Sint Franciscus Hospital, IJsselland Hospital, Reinier de Graaf Hospital, Elisabeth-Tweesteden hospital, Albert Schweitzer Hospital). Most patient data and samples used in the studies in this thesis, are enrolled from the MATCH study, unless stated otherwise. With the availability of the MATCH data, recurrence and mortality rates can be studied in a well-defined population helping to clarify differences in treatment and outcome between patients and hospitals.

Daily practice

Differences in patient- and tumor characteristics and variance in prognosis necessitate a personalized approach. Surgical resection of the primary colon tumor is considered the backbone for patients with stage I-III disease. For patients with lymph node involvement (stage III), standard treatment consists of surgical resection followed by adjuvant systemic chemotherapy. Multiple large randomized controlled trials (RCTs) have been performed to investigate which chemotherapy is most effective in colon cancer patients [10-19]. The current adjuvant systemic chemotherapy according to (inter)national guidelines consists of a fluoropyrimidine, either in combination with oxaliplatin, or as monotherapy. Numerous studies have confirmed the survival benefit of this adjuvant treatment [14, 18, 20, 21]. Since then, few alterations have been made in the adjuvant treatment due to limited effect of newly developed therapeutics and often disappointing results on recurrence, survival and toxicity. Although the oxaliplatin-based regimens provide the best outcomes regarding survival, it also leads to substantial toxicity with sometimes irreversible neuropathy and a negative impact on quality of life. In attempts to de-escalate chemotherapy and thereby reduce toxicity, the IDEA-study was conducted [22, 23]. In this preplanned pooled analysis of six RCTs, 3 months of adjuvant fluoropyrimidine and oxaliplatin-based chemotherapy was compared with 6 months in patients with stage III colon cancer. The authors conclude: “Although noninferiority of 3 months versus 6 months of adjuvant chemotherapy for patients with stage III colon cancer was not confirmed in terms of overall survival, the absolute 0.4% difference in 5-year overall survival should be placed in clinical context. Overall survival results support the use of 3 months of adjuvant CAPOX for most patients with stage III colon cancer. This conclusion is strengthened by the substantial

reduction of toxicities, inconveniencies, and cost associated with a shorter treatment duration." [22, 23]. These results were a major milestone and led to the recommendation of 3 months CAPOX and 6 months FOLFOX as adjuvant chemotherapy regimens for stage III colon cancer in international guidelines [24]. In order to further optimize treatment for colon cancer patients, we questioned how current patient selection for adjuvant treatment can be improved, as individual differences in clinical outcome within a single tumor stage still remain [25].

Biomarkers

Besides aforementioned patient factors and TNM-staging, molecular characteristics of tumors have been shown to have an impact on prognosis and prediction. A prognostic biomarker provides information about the patients overall cancer outcome, regardless of therapy. The presence or the absence of such a prognostic marker can be useful for the selection of patients for a certain treatment, but does not predict the response to this treatment. A predictive biomarker provides information on the effect of a therapeutic intervention in a patient [26].

Additional biomarkers to classify patients at risk of recurrence are needed, as 20 percent of low-risk patients (stage I/II) develops recurrent disease despite surgical resection with curative intent. Therefore, efforts are being directed at finding so far unknown factors that may play a role in the development and progression of colon cancer [27]. Factors influencing cancer progression can be found in genetic and epigenetic processes. Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product that enables it to produce a protein as the end product [28]. Transcription of DNA into RNA molecules is the first step in gene-expression. Besides segments of DNA that are transcribed into proteins (messenger RNA, mRNA), segments of DNA transcribed into RNA molecules that do not encode proteins are called non-coding RNAs (ncRNAs). Transcriptome sequencing studies have identified many short and long RNAs with non-protein-coding ability [29-32]. Recent studies have shown that these ncRNAs can function as promising prognostic biomarkers for stage I-II colon cancer patients [27, 33, 34]. Among ncRNAs, circular RNA (circRNAs) represents an molecule with a unknown mechanism of action. Normally, RNA polymerase builds RNA strands in the 5' to 3' direction, forming linear RNA. However, in circular RNA the 5' and 3' ends have been joined together, forming covalently closed, continuous loop structures (Figure 1) [35-37].

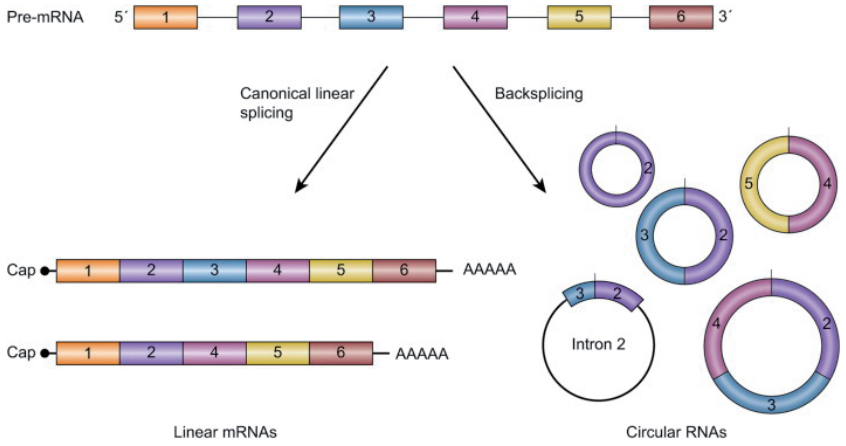

Figure 1. Biogenesis of circular RNA
(adapted from Huang et al. 2017 [38])

Circular RNAs have been proven to be stably present in saliva, blood, and exosomes, which makes them promising biomarkers for the diagnosis, prognosis, and therapeutic assessment of cancer patients [39]. Recently, novel circRNAs were identified as prognostic biomarkers for stage II colon cancer patients [40]. These characteristics may indicate that circRNAs could represent new clinical diagnostic and prognostic markers for colon cancer patients.

Consensus Molecular Subtypes

Although many studies provide valuable information on prognostic biomarkers, we are currently still not capable of adequate upfront selection of high risk colon cancer patients who may benefit from chemotherapy. This stresses the need for additional upfront predictive markers for response to chemotherapy. Many efforts have been undertaken to stratify colon cancer patients by other means than TNM-staging, into biologically and clinically distinct subtypes. One of these approaches led to the development of the Consensus Molecular Subtypes (CMSs) (Figure 2) [41].

CMS1 MSI immune	CMS2 Canonical	CMS3 Metabolic	CMS4 Mesenchymal
14%	37%	13%	23%
MSI, CIMP high, hypermutation	SCNA high	Mixed MSI status, SCNA low, CIMP low	SCNA high
BRAF mutations		*KRAS* mutations	
Immune infiltration and activation	WNT and MYC activation	Metabolic deregulation	Stromal infiltration, TGF-β activation, angiogenesis
Worse survival after relapse			Worse relapse-free and overall survival

Figure 2. Consensus molecular subtypes with distinct biological characteristics
(adapted from Guinney et al. 2015 [41])
Abbreviations: MSI=microsatellite instability; CIMP= Island methylator phenotype, SCNA = Somatic copy number alterations.

The CMS classification divides colon cancer patients into four subtypes with distinctive biological features. CMS1 is characterized by hypermutation, microsatellite instability (MSI) and strong immune infiltration. CMS2, the canonical subtype, has marked WNT and MYC signaling activation. CMS3 is enriched for *KRAS*-mutations and shows evident metabolic deregulation. CMS4, the mesenchymal subtype, is characterized by prominent TGF-β activation, stromal invasion and angiogenesis activation. Due to the differences in biological characteristics and molecular composition of these four subtypes, the prognosis for these subtypes is significantly different [41]. CMS seems to have a prognostic value as well as predictive for response to systemic treatment [42, 43]. The gold-standard classification strategy for CMS relies on genome-wide RNA expression data from fresh-frozen tumor tissue. Since fresh-frozen tissue sampling is not standard during surgical resection, this hampers widespread implementation of the classification. Therefore, the need for a more practical, minimally-invasive test to distinguish between subtypes remains. As can be noted in Figure 2, characteristics that differentiate between CMS subtypes are CIMP-high or low, MSI and hypermutation. These characteristics are the result of epigenetic alterations caused by methylation. CMS subtypes exhibit different methylation profiles and might therefore be distinguishable based on these profiles. DNA methylation markers are detectable in minimally-invasive bodily fluids, stool, as well as formalin-fixed paraffin embedded tissue which could make them useful for easy classification.

OUTLINE OF THIS THESIS

The general aim of this thesis was to address the challenging aspects in daily practice regarding prognosis and treatment for stage I-III colon cancer patients. **Chapter 2** presents a systematic review assessing RCTs on the different systemic treatment modalities for colon cancer patients. The study gives an overview of numerous performed and currently ongoing studies on systemic treatment and gives insights in molecular markers for patient selection for systemic therapy. In **chapter 3,** we analyzed data from patients from the MATCH study who had at least 5 years follow up and characterized patients who survived up to five years without recurrence and identified factors predicting the probability of long-term recurrence free survival. In **chapter 4**, we analyzed data from all colon cancer patients in the MATCH study and investigated whether treatment variation exists between the participating hospitals and if survival outcomes were different between these centers. In the current era of personalized treatment, analyzing differences in daily practice and identification of the impact of treatment decisions is of utmost importance. Literature suggests that many eligible patients do not receive adjuvant chemotherapy, for various reasons and the underlying reasons for this omission may vary strongly. Therefore, in **chapter 5,** we investigated these reasons for guideline non-adherence regarding administration of adjuvant chemotherapy and assessed the effect on patient outcomes for those receiving surgery plus adjuvant chemotherapy versus surgery alone. Next to differences in treatment, specific patient factors are associated with survival. Among these patient factors, socioeconomic status is gaining increasing attention in relation to outcomes of various cancers. We investigated whether socioeconomic status is also associated with short and long-term outcome in patients undergoing curative surgery for CRC in **chapter 6**. Therewithal, molecular characteristics of tumors have been shown to have an impact on prognosis and prediction as well. Identifying the patients at risk of developing metastases, as well as those responding to therapy is a clear unmet need in colon cancer care. Additional biomarkers to classify patients at risk of recurrence are needed. In **chapter 7** we studied the feasibility to identify an extensive catalog of circular RNAs in chemonaive, low risk (stage I/II) colon cancer patients and related circRNA expression to clinical outcome, microsatellite status and CMS. These consensus molecular subtypes have been developed to better guide precision treatment. The gold-standard classification strategy relies on genome-wide RNA expression data from sufficient quantities of fresh-frozen bulk tumor, which hampers widespread implementation. To advance this classification method into clinical practice, a highly reliable and practical classification method is needed. In **chapter 8,** we identified methylation markers to distinguish between CMS2 and 3 in patients with CRC for whom an easy test is currently lacking. In spite of all these efforts, we are currently, still not capable of upfront selection of high risk colon cancer patients who may benefit from chemotherapy. Therefore, we have set up a national, prospective, single arm, multicenter intervention study investigating whether the CMS classification can be of added value in clinical decision making

by analyzing the predictive value of neoadjuvant chemotherapy in high-risk stage II and stage III colon cancer patients (CONNECTION-II study). In **chapter 9**, we describe the study protocol for the CONNECTION-II study.

The treatment and outcomes for patients with colon cancer vary significantly. In this thesis, we have attempted to investigate different facets related to outcome. By analyzing patient-related factors such as SES, between-hospitals differences, daily practice regarding systemic treatment, and research into biological tumor characteristics, we aimed to obtain insight into factors that are associated with, and contribute to, the disparity in outcomes between seemingly comparable patients.

REFERENCES

1. Nederlandse Kankerregistratie (NKR), IKNL.
2. TNM Classification of Malignant Tumours, 8th Edition. In: James D. Brierley MKG, Christian Wittekind ed. TNM Classification of Malignant Tumours, 8th Edition: Wiley-Blackwell; 2016.
3. Karahalios A, English DR, Simpson JA. Weight change and risk of colorectal cancer: a systematic review and meta-analysis. *Am J Epidemiol* 2015; 181(11):832-45.
4. Lauby-Secretan B, Scoccianti C, Loomis D, et al. Body Fatness and Cancer--Viewpoint of the IARC Working Group. *N Engl J Med* 2016; 375(8):794-8.
5. Johnson CM, Wei C, Ensor JE, et al. Meta-analyses of colorectal cancer risk factors. *Cancer Causes Control* 2013; 24(6):1207-22.
6. Yuhara H, Steinmaus C, Cohen SE, et al. Is diabetes mellitus an independent risk factor for colon cancer and rectal cancer? *Am J Gastroenterol* 2011; 106(11):1911-21; quiz 1922.
7. Coebergh van den Braak RRJ, Lalmahomed ZS, Buttner S, et al. Nonphysician Clinicians in the Follow-Up of Resected Patients with Colorectal Cancer. *Dig Dis* 2018; 36(1):17-25.
8. Lalmahomed ZS, Coebergh van den Braak RRJ, Oomen MHA, et al. Multicenter fresh frozen tissue sampling in colorectal cancer: does the quality meet the standards for state of the art biomarker research? *Cell Tissue Bank* 2017; 18(3):425-431.
9. Kloosterman WP, Coebergh van den Braak RRJ, Pieterse M, et al. A systematic analysis of oncogenic gene fusions in primary colon cancer. *Cancer Res* 2017.
10. Twelves C, Scheithauer W, McKendrick J, et al. Capecitabine versus 5-fluorouracil/folinic acid as adjuvant therapy for stage III colon cancer: Final results from the X-ACT trial with analysis by age and preliminary evidence of a pharmacodynamic marker of efficacy. *Ann Oncol* 2012; 23(5):1190-1197.
11. Shimada Y, Hamaguchi T, Mizusawa J, et al. Randomised phase III trial of adjuvant chemotherapy with oral uracil and tegafur plus leucovorin versus intravenous fluorouracil and levofolinate in patients with stage III colorectal cancer who have undergone Japanese D2/D3 lymph node dissection: Final results of JCOG0205. *Eur J Cancer* 2014; 50(13):2231-2240.
12. De Gramont A, Henriques J, Baruch B, et al. Bevacizumab plus oxaliplatin-based chemotherapy as adjuvant treatment for colon cancer (CC): Updated analysis of stage II disease from the AVANT phase III randomized trial by the GERCOR group. *Ann Oncol* 2019; 30:v209.
13. de Gramont A, Van Cutsem E, Schmoll HJ, et al. Bevacizumab plus oxaliplatin-based chemotherapy as adjuvant treatment for colon cancer (AVANT): A phase 3 randomised controlled trial. *Lancet Oncol* 2012; 13(12):1225-1233.
14. Haller DG, Tabernero J, Maroun J, et al. Capecitabine plus oxaliplatin compared with fluorouracil and folinic acid as adjuvant therapy for stage III colon cancer. *J Clin Oncol* 2011; 29(11):1465-1471.
15. Pectasides D, Karavasilis V, Papaxoinis G, et al. Randomized phase III clinical trial comparing the combination of capecitabine and oxaliplatin (CAPOX) with the combination of 5-fluorouracil, leucovorin and oxaliplatin (modified FOLFOX6) as adjuvant therapy in patients with operated high-risk stage II or stage III colorectal cancer. *BMC Cancer* 2015; 15(1).
16. Kuebler JP, Wieand HS, O'Connell MJ, et al. Oxaliplatin combined with weekly bolus fluorouracil and leucovorin as surgical adjuvant chemotherapy for stage II and III colon cancer: results from NSABP C-07. *J Clin Oncol* 2007; 25(16):2198-204.

17. Yothers G, O'Connell MJ, Allegra CJ, et al. Oxaliplatin as adjuvant therapy for colon cancer: updated results of NSABP C-07 trial, including survival and subset analyses. *J Clin Oncol* 2011; 29(28):3768-74.
18. André T, Boni C, Navarro M, et al. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. *J Clin Oncol* 2009; 27(19):3109-3116.
19. Andre T, Boni C, Mounedji-Boudiaf L, et al. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. *N Engl J Med* 2004; 350(23):2343-51.
20. Labianca R, Nordlinger B, Beretta GD, et al. Early colon cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2013; 24 Suppl 6:vi64-72.
21. Moertel CG, Fleming TR, Macdonald JS, et al. Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. *N Engl J Med* 1990; 322(6):352-8.
22. Andre T, Meyerhardt J, Iveson T, et al. Effect of duration of adjuvant chemotherapy for patients with stage III colon cancer (IDEA collaboration): final results from a prospective, pooled analysis of six randomised, phase 3 trials. *Lancet Oncol* 2020; 21(12):1620-1629.
23. Grothey A, Sobrero AF, Shields AF, et al. Duration of Adjuvant Chemotherapy for Stage III Colon Cancer. *N Engl J Med* 2018; 378(13):1177-1188.
24. Argiles G, Tabernero J, Labianca R, et al. Localised colon cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2020; 31(10):1291-1305.
25. Dienstmann R, Mason MJ, Sinicrope FA, et al. Prediction of overall survival in stage II and III colon cancer beyond TNM system: a retrospective, pooled biomarker study. *Ann Oncol* 2017; 28(5):1023-1031.
26. Oldenhuis CN, Oosting SF, Gietema JA, et al. Prognostic versus predictive value of biomarkers in oncology. *Eur J Cancer* 2008; 44(7):946-53.
27. Jacob H, Stanisavljevic L, Storli KE, et al. A four-microRNA classifier as a novel prognostic marker for tumor recurrence in stage II colon cancer. *Sci Rep* 2018; 8(1):6157.
28. Crick FH. On protein synthesis. *Symp Soc Exp Biol* 1958; 12:138-63.
29. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* 2009; 10(1):57-63.
30. Iyer MK, Niknafs YS, Malik R, et al. The landscape of long noncoding RNAs in the human transcriptome. *Nat Genet* 2015; 47(3):199-208.
31. Muers M. RNA: Genome-wide views of long non-coding RNAs. *Nat Rev Genet* 2011; 12(11):742.
32. Sanger HL, Klotz G, Riesner D, et al. Viroids are single-stranded covalently closed circular RNA molecules existing as highly base-paired rod-like structures. *Proc Natl Acad Sci U S A* 1976; 73(11):3852-6.
33. Bullock MD, Pickard K, Mitter R, et al. Stratifying risk of recurrence in stage II colorectal cancer using deregulated stromal and epithelial microRNAs. *Oncotarget* 2015; 6(9):7262-79.
34. Grassi A, Perilli L, Albertoni L, et al. A coordinate deregulation of microRNAs expressed in mucosa adjacent to tumor predicts relapse after resection in localized colon cancer. *Mol Cancer* 2018; 17(1):17.
35. Hsu MT, Coca-Prados M. Electron microscopic evidence for the circular form of RNA in the cytoplasm of eukaryotic cells. *Nature* 1979; 280(5720):339-40.
36. Wilusz JE, Sharp PA. Molecular biology. A circuitous route to noncoding RNA. *Science* 2013; 340(6131):440-1.

37. Cocquerelle C, Mascrez B, Hetuin D, et al. Mis-splicing yields circular RNA molecules. *FASEB J* 1993; 7(1):155-60.
38. Huang S, Yang B, Chen BJ, et al. The emerging role of circular RNAs in transcriptome regulation. *Genomics* 2017; 109(5-6):401-407.
39. Zhang M, Xin Y. Circular RNAs: a new frontier for cancer diagnosis and therapy. *J Hematol Oncol* 2018; 11(1):21.
40. Karousi P, Artemaki PI, Sotiropoulou CD, et al. Identification of Two Novel Circular RNAs Deriving from BCL2L12 and Investigation of Their Potential Value as a Molecular Signature in Colorectal Cancer. *Int J Mol Sci* 2020; 21(22).
41. Guinney J, Dienstmann R, Wang X, et al. The consensus molecular subtypes of colorectal cancer. *Nat Med* 2015; 21(11):1350-6.
42. Song N, Pogue-Geile KL, Gavin PG, et al. Clinical Outcome From Oxaliplatin Treatment in Stage II/III Colon Cancer According to Intrinsic Subtypes: Secondary Analysis of NSABP C-07/NRG Oncology Randomized Clinical Trial. *JAMA Oncol* 2016; 2(9):1162-9.
43. Roepman P, Schlicker A, Tabernero J, et al. Colorectal cancer intrinsic subtypes predict chemotherapy benefit, deficient mismatch repair and epithelial-to-mesenchymal transition. *Int J Cancer* 2014; 134(3):552-62.

Chapter 2

A Decade of Systemic Treatment for Stage II-III Colon Cancer Patients: What has changed and what's to come? A systematic review of the randomized trials

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Submitted

ABSTRACT

Introduction

This review gives an overview of the current trends in (neo)adjuvant therapy and patient selection for systemic treatment in stage II-III colon cancer (CC) patients.

Methods

A systematic literature search was performed. We included randomized controlled trial (RCTs) published from 2010 onwards and complemented the search by including currently ongoing RCTs on systemic treatment and patient selection by molecular testing.

Results

The efficacy of FOLFOX and CAPOX was confirmed in multiple RCTs but no new systemic therapies were validated. Multiple RCTs studying different monotherapy schedules of fluoropyrimidines showed equivalent effect in stage III patients. Survival results from the IDEA-study showed that 3 months of adjuvant CAPOX was non-inferior to 6 months for most stage III patients. Neoadjuvant immunotherapy has shown promising results with the potential to become the new standard of care for a specific patient group.

Conclusion

The only significant progress made in systemic treatment the last decade is reducing the duration of adjuvant chemotherapy from 6 to 3 months in stage III patients. No RCTs on new agents showed survival benefit over conventional therapies. Knowledge about prognosis and prediction has improved by molecular analyses. However, validation in RCTs of added value of biomarkers is lacking. It is essential that promising developments such as neoadjuvant treatment, immunotherapy for MSI and ctDNA are studied in RCTs followed by validation to be able to implement these in daily practice. Given the increasing number of CC subgroups resulting in smaller patient numbers, international collaborations such as the IDEA and FOxTROT are paramount.

INTRODUCTION

Colorectal cancer (CRC) is one of the most common types of cancer with an incidence of around 1.9 million worldwide in 2020 [1]. Surgical resection of the primary tumor is considered the backbone of treatment for patients with stage I-III colon cancer with a 5-year survival rate between 63 and 92% [2]. For patients with high risk stage II and stage III colon cancer, standard treatment following surgical resection consists of adjuvant systemic chemotherapy with a fluoropyrimidine either in combination with oxaliplatin or as monotherapy. The doublet schedule with oxaliplatin is based on studies published at least 10 years ago [3-8]. Although the oxaliplatin-based regimens provide the best outcomes regarding disease free survival (DFS) and overall survival (OS), it also leads to substantial toxicity with sometimes irreversible neuropathy and a negative impact on quality of life. In attempts to reduce this toxicity, the IDEA-study was conducted in which 3 months of adjuvant fluoropyrimidine plus oxaliplatin was compared with 6 months in patients with stage III colon cancer. Results from this study led to a reduction in duration to 3 months of adjuvant chemotherapy for a subgroup of stage III patients resulting in significantly fewer side effects [9].

Despite the current available knowledge and experience, 30-35% of the patients treated with adjuvant chemotherapy will still develop metastatic disease while on estimate 50% of these patients would have remained disease-free without adjuvant treatment and thus are over-treated [10, 11]. Unfortunately, we are not yet able to identify patients upfront who would benefit most of this adjuvant therapy.

New treatment regimens have been assessed in the last decade with varying and often disappointing results on recurrence, survival and toxicity. However, alterations have been made regarding the eligibility of patients for adjuvant treatment, mostly as a result of adjusted definitions of high-risk stage II disease. Until recently, high-risk features for stage II disease included high histological grade, lymphovascular or perineural invasion, mucinous component, T4 stage, extramural vein invasion, symptomatic bowel obstruction or perforation at diagnosis and less than 12 lymph nodes removed. These high risk features are still under debate, resulting in different guidelines with conflicting advices. Molecular biomarkers that provide prognostic or predictive information regarding response to treatment, enables clinicians to make decisions between different treatment options whilst avoiding toxicity in patients unlikely to gain therapeutic benefit. For example, we know that patients with deficient mismatch repair (dMMR) tumors in early stage disease have a better prognosis compared with patients with proficient mismatch repair tumors (pMMR) [5]. In addition, it has also been observed that dMMR predicts lack of response to fluoropyrimidine-monotherapy in the adjuvant setting [12, 13]. Many studies have been conducted over the last years aiming towards new treatment options, better upfront selection for systemic treatment and thereby possible de-escalating chemotherapy in some patients. This review gives an overview of the evidence from previously performed and currently ongoing randomized controlled trials (RCTs) regarding systemic treatment and patient selection for systemic therapy in stage II-III colon cancer patients.

METHODS

This review was conducted and reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).

Literature search and study selection

A systematic literature search was performed with the help of a clinical librarian (WMB) using descriptors that included synonyms for 'colon cancer' and 'adjuvant chemotherapy' in various combinations (Table 1).

The search was conducted in Embase.com, Medline (Ovid) and Cochrane Central and included all articles published from 2010 until February 2021. Studies were evaluated for inclusion by two independent reviewers (IB and RRJC). Articles were screened based on title and abstract and

Table 1. Search terms per database

Database	Search terms
Embase.com	('large intestine tumor'/de OR 'colon tumor'/exp OR 'large intestine cancer'/exp OR 'rectum tumor'/exp OR 'colorectal cancer cell line'/exp OR (((colon OR colonic OR sigmoid* OR 'large intestin*' OR colorectal* OR rectal OR rectum) NEAR/6 (cancer* OR carcino* OR adenocarcino* OR adenoma* OR neoplas* OR tumo*))):ab,ti) AND ('adjuvant therapy'/exp OR 'neoadjuvant therapy'/exp OR 'molecular therapy'/exp OR 'molecular marker'/exp OR (((adjuvant* OR neoadjuvant*) NEAR/3 (therap* OR chemotherap* OR treat*)) OR ((target* OR gene OR enzyme*) NEAR/3 (therap* OR agent* OR molecular* OR anticancer* OR anti-cancer*)) OR ((molecul*) NEAR/3 (marker* OR biomarker*))):ab,ti) AND ('high risk patient'/de OR ((locally NEAR/3 advance*) OR non-metastat* OR nonmetastat* OR (stage* NEXT/1 (3 OR iii)) OR 'high* risk*'):ab,ti) AND [english]/lim ('Controlled clinical trial'/exp OR 'Crossover procedure'/de OR 'Double-blind procedure'/de OR 'Single-blind procedure'/de OR (random* OR factorial* OR crossover* OR (cross NEXT/1 over*) OR placebo* OR ((doubl* OR singl*) NEXT/1 blind*) OR assign* OR allocat* OR volunteer* OR trial OR groups):ab,ti,kw) NOT ([animals]/lim NOT [humans]/lim)
Medline	(Colorectal Neoplasms/ OR exp Colonic Neoplasms/ OR Rectal Neoplasms/ OR (((colon OR colonic OR sigmoid* OR large intestin* OR colorectal* OR rectal OR rectum) ADJ6 (cancer* OR carcino* OR adenocarcino* OR adenoma* OR neoplas* OR tumo*))).ab,ti.) AND (exp Chemotherapy, Adjuvant/ OR Neoadjuvant Therapy/ OR Molecular Targeted Therapy/ OR (((adjuvant* OR neoadjuvant*) ADJ3 (therap* OR chemotherap* OR treat*)) OR ((target* OR gene OR enzyme*) ADJ3 (therap* OR agent* OR molecular* OR anticancer* OR anti-cancer*)) OR (molecul* ADJ3 (marker* OR biomarker*))).ab,ti.) AND (((locally ADJ3 advance*) OR non-metastat* OR nonmetastat* OR (stage* ADJ ("3" OR iii)) OR high* risk*).ab,ti.) AND english.la. AND (Exp Controlled clinical trial/ OR "Double-Blind Method"/ OR "Single-Blind Method"/ OR "Random Allocation"/ OR (random* OR factorial* OR crossover* OR cross over* OR placebo* OR ((doubl* OR singl*) ADJ blind*) OR assign* OR allocat* OR volunteer* OR trial OR groups).ab,ti,kf.) NOT (exp Animals/ NOT Humans/)
Cochrane	((((colon OR colonic OR sigmoid* OR large-intestin* OR colorectal* OR rectal OR rectum) NEAR/6 (cancer* OR carcino* OR adenocarcino* OR adenoma* OR neoplas* OR tumo*))):ab,ti) AND ((((adjuvant* OR neoadjuvant*) NEAR/3 (therap* OR chemotherap* OR treat*)) OR ((target* OR gene OR enzyme*) NEAR/3 (therap* OR agent* OR molecular* OR anticancer* OR anti-cancer*)) OR ((molecul*) NEAR/3 (marker* OR biomarker*))):ab,ti) AND ((((locally NEAR/3 advance*) OR non-metastat* OR nonmetastat* OR (stage* NEXT/1 (3 OR iii)) OR 'high* risk*'):ab,ti)

if the eligibility criteria were met, full manuscripts were procured and reviewed. Disagreements were resolved by discussion. Inclusion criteria were RCTs, of which the manuscript was written in English, including patients diagnosed with stage II or stage III colon cancer and reporting on outcome of (neo)adjuvant chemotherapy. Studies published before 2010 or describing tumors other than colon cancer were excluded in addition to non-RCTs. We also excluded studies lacking details on enrolment in order to prevent reporting on overlapping data.

In addition to the systematic search, we examined reference lists from included articles for suitable studies. To give an overview of the current trends in systemic treatment and future directions, we complemented the studies from our literature search by including currently ongoing RCTs on systemic therapy and patient selection for systemic treatment.

RESULTS

Adjuvant Chemotherapy (Table 2)

Intravenous versus oral chemotherapy

Fluoropyrimidines (either intravenous fluorouracil or oral capecitabine) have been the conventional chemotherapy agents for stage II-III colon cancer patients for over 30 years. Fluorouracil is administered in combination with leucovorin, a folic acid derivative that potentiates the cytotoxic inhibitory effects of fluorouracil. The X-ACT trial [14], JCOG0205 [15], NO16968 [4], and ACTRN 12610 [16] compared a minimum of 6 months treatment with oral versus intravenous fluoropyrimidines, of which the first 3 trials included only stage III patients. Long-term results of the X-ACT trial [14] showed that oral capecitabine is at least equivalent to an intravenous bolus regimen of 5-fluorouracil/Leucovorin (5-FU/LV) (Mayo Clinic regimen) with regard to 5-year DFS and 5-year OS. Furthermore, the study showed that patients in the capecitabine group experienced significantly less side effects. On the basis of the X-ACT trial, capecitabine was approved as monotherapy for the adjuvant treatment in stage III colon cancer patients. The JCOG0205 trial showed equivalent 5-year DFS and 5-year OS of oral uracil-tegafur/leucovorin (UFT/LV) as compared with 5-FU/LV with comparable side-effects in both groups [15]. The NO16968 and ACTRN 12610 studies included combination schedules. Long-term results of the NO16968 trial showed improved 5-year DFS and 5-year OS for treatment with CAPOX compared with 5-FU/LV (Mayo Clinic or Roswell Park regimen) [4]. The ACTRN 12610 trial included both high risk stage II and stage III colon cancer patients. High-risk features for stage II disease were defined as either high histological grade, lymphovascular or perineural invasion, mucinous component, T4 stage, extramural vein invasion, symptomatic bowel obstruction or perforation at diagnosis and less than 12 lymph nodes removed. Results showed equal 3-year DFS and 5-year OS across both treatment arms comparing CAPOX and FOLFOX [16].

Table 2. Studies on adjuvant chemotherapy

Study Identifier (Acronym)	Author	No. of patients	Enrolment	Interventions	Outcome	Length of follow-up
Completed trials						
Intravenous versus oral chemotherapy						
X-act trial	Twelvers 2011 [14]	1987	Stage III colon cancer patients, Nov 1998 – Nov 2001	Arm 1: Capecitabine Arm 2: 5-FU/LV	5-year DFS 5-year OS	Median follow up 6.9 years
JCOG0205	Shimada 2014 [15]	1101	Stage III colon cancer patients, Feb 2003 – Nov 2006	Arm 1: 5-FU/LV Arm 2: UFT/LV	5-year DFS 5-year OS	Median follow-up 72.0 months
NO16968	Schmoll 2015 [87]	1886	Stage III Colon cancer patients, April 2003 and Oct 2004.	Arm 1: XELOX Arm 2: 5-FU/LV	DFS, OS	Median follow-up 7 years
ACTRN 12610	Pectasides 2015 [16]	441	Stage II-III colon cancer patients, Nov 2005 – Jan 2008	Arm 1: FOLFOX6 Arm 2: CAPOX	3-year DFS 5-year OS	Median follow-up of 74.7 month
Oral chemotherapy regimens						
ACTS-CC trial	Kusumoto 2018 [19]	1518	Stage III colon cancer patients, April 2008 - June 2009	Arm 1: S-1 Arm 2: UFT/LV	3 year DFS	Median follow-up 63.5 months
ACTS-CC 02 trial	Sunami 2020 [20]	966	Stage III colon cancer patients, April 2010 - Oct 2014	Arm 1: S-1 + oxaliplatin (SOX) Arm 2: UFT/LV	3-year DFS	Median follow-up 58.4 months
JCOG0910	Hamaguchi 2018 [21]	1,564	Stage III colon cancer patients, March 2010 - Aug 2013	Arm 1: capecitabine Arm 2: S-1	3-year DFS	Median follow-up 4.13 year
Single fluoropyrimidines versus combination schedules						
MOSAIC	Andre 2015 [88]	2246	Stage II-III colon cancer patients, Oct 1998 - Jan 2001	Arm 1: 5-FU/LV Arm 2: FOLFOX	10 year OS 10 year DFS	Median follow up 9.5 years
NO16968	Schmoll 2015 [87]	1886	Stage III Colon cancer patients, April 2003 and Oct 2004	Arm 1: XELOX Arm 2: 5-FU/LV	5-year DFS 5-year OS	Median follow-up almost 7 years
	Papadimitriou 2011 [24]	873	Stage III colon cancer patients, Jan 1999 -Sept 2004	Arm 1: IFL Arm 2: 5-FU/LV	3-year DFS 3-year OS	Median follow-up 77.4 months

Table 2. Studies on adjuvant chemotherapy (continued)

	Paschke 2019 [25]	275	stage IIb and III colon cancer patients, Jan 2002 - Jan 2008	Arm 1: 5-FU/LV Arm 2: FOLFIRI	5-year OS	Median follow-up 4.92 years
Currently ongoing						
Study Identifier (Acronym)	**Author**	**Target no. of patients**	**Enrolement**	**Interventions**	**Outcome**	
IROCAS	Bennauna et al. 2019 [89]	640	pT4N1 or pT1-4N2 colon cancer patients	Arm 1: adjuvant mFOLFIRINOX Arm 2: mFOLFOX6	DFS	

Abbreviations: LV: Leucovorin calcium, FA folinic acid, 5-FU: 5-fluorucacil, s-1: cisplatin, UFT: Tegafur and uracil, FOLFOX: leucovorin calcium (folinic acid) & fluorouracil & oxaliplatin, CAPOX: capecitabine & oxaliplatin, XELOX: capecitabine & oxaliplatin, FOLFORI: 5-FU/LV + irinotecan, IFL: 5-FU (bolus)/LV + irinotecan FOLFIRINOX:FOLFOX + irinotecan

Oral chemotherapy regimens

S-1 is an oral fluoropyrimidine that combines the 5-FU prodrug tegafur with oteracil and gimeracil. This regimen has some potential advantages over capecitabine including lower frequency of hand–foot syndrome which was demonstrated in metastatic CRC patients [17, 18]. Three trials were performed comparing 6 months tegafur-oteracil-gimeracil (S-1) with established oral regimens in patients with stage III colon cancer. The ACTS-CC trial, compared S-1 with UFT/LV and in the ACTS-CC 02 trial, oxaliplatin was added to S-1 and compared with UFT/LV [19, 20]. Both trials concluded that adjuvant therapy with S-1 (both with and without oxaliplatin) was non-inferior in 3-year DFS to treatment with UFT/LV. No reduction in side-effects was observed from treatment with S1 relative to UFT/LV. In the ACTS-CC 02 study, a higher rate of side-effects was found in the S-1 group as compared with the UFT/LV group.

The JCOG0910 study was terminated prematurely after the second interim analysis failed to show non-inferiority of adjuvant S-1 to capecitabine in DFS. The updated follow-up data confirmed the conclusion from the interim analysis [21]. Grade 2 or higher hand-food syndrome was observed less frequent in the S-1 group compared with capecitabine.

Monotherapy versus combination schedules with fluoropyrimidines

The ten-year results of the MOSAIC trial confirmed the OS benefit from 6 months adjuvant FOLFOX versus 5-FU/LV in patients with stage III colon cancer, but showed no benefit in patients with high risk stage II disease, defined as either pT4, tumor perforation, or fewer than 10 lymph nodes examined [22, 23]. Similarly, as mentioned previously, long-term results of the NO16968 trial showed improved 5-year DFS and 5-year OS for treatment with CAPOX compared with 5-FU/LV [4]. Results of both studies demonstrated that addition of oxaliplatin increased toxicity.

In the last ten year, two trials were published comparing 5-FU/LV with -5FU/LV + irinotecan [24, 25]. One study used the bolus 5FU + LV + irinotecan regimen (IFL) REF, the other study used the same composition but with a 24 hour 5-FU administration per automatic pump system (FOLFIRI) instead of bolus administration of 5-FU. Results of both studies clearly demonstrated that addition of irinotecan markedly increased toxicity, and did neither reduce the frequency of recurrences nor enhance 5-year OS in the whole cohort. Currently, the IROCAS-study is ongoing to analyze whether the addition of irinotecan to FOLFOX (FOLFIRINOX), does improve DFS in colon cancer patients with a pT4 and/or N2 tumor [26]

Targeted therapies (Table 3)

Four studies investigated the addition of targeted therapies to doublet chemotherapy (FOLFOX/CAPOX) in patients with stage III colon cancer. Two out of these four studies, (N0147 and PETACC-8 study) investigated the effect of adding an anti-epidermal growth factor receptor (eGFR) antibody (cetuximab) to standard FOLFOX for resected stage III colon cancer patients [27, 28]. Both studies were amended to restrict inclusion to wild-type KRAS tumors. The N0147 study had to terminate recruitment prematurely after a second interim analysis which demonstrated no benefit when adding cetuximab to FOLFOX. DFS at 3 years was 71.5% for FOLFOX plus cetuximab and 74.6% for FOLFOX alone, even suggesting a trend towards harm with the addition of cetuximab. None of the subgroup analyses showed survival benefit from cetuximab while grade 3 or higher adverse events were significantly more frequent. The PETACC-8 study also showed that the addition of cetuximab to FOLFOX did not improve 3-year DFS compared with FOLFOX.

The AVANT trial and the NSABP C-08 trial both investigated the addition of anti-vascular endothelial growth factor (VEGF) antibody (bevacizumab) to doublet chemotherapy [29, 30 31]. The NSABP-C08 study tested the addition of bevacizumab to FOLFOX in both stage II and III colon cancer patients and showed no improvement in 3-year DFS compared to FOLFOX alone. Similarly, the AVANT-trial concluded that addition of bevacizumab did not prolong 3-year DFS, with even a potential detrimental effect on 5-year OS, in stage III patients.

Post-hoc analyses from the NSABP-C08 trial showed that patients diagnosed with dMMR tumors derived statistically significant survival benefit from the addition of bevacizumab in contrast with no benefit in patients diagnosed with pMMR tumors [32]. Since these results were obtained by post-hoc analyses, the original trial was not powered for these analyses. Independent validation in other RCTs is needed to confirm this finding.

The use of selective cyclooxygenase 2 (COX-2) inhibitors has been associated with a possible lower risk of recurrent disease in colon cancer patients. In the Alliance trial stage III colon cancer patients were randomized to receive adjuvant FOLFOX with or without a COX-2 inhibi-

Table 3. Studies on targeted therapies

Study Identifier (Acronym)	Author	No. of patients	Enrolment	Interventions	Outcome	Length of follow-up
Completed trials						
[NCCTG] N0147	Alberts et al. 2012 [27]	2686	Stage III colon cancer patients, Feb 2004 – Nov 2009	Arm 1: mFOLFOX6 Arm 2.: mFOLFOX 6 + cetuximab	3-year DFS	Median follow-up 28 months
PETACC-8	Taieb et al.2014 [28]	2559	Stage III colon cancer patients, Dec 2005- Nov 2009	Arm 1: FOLFOX4 Arm 2: FOLFOX4 + cetuximab	3-year DFS	Median follow-up 3·3 years
AVANT	De Gramont et al.2012 [30]	3451	Stage II-III Colon cancer patients, Dec 2004- June 2007	Arm 1: FOLFOX4 Arm 2: FOLFOX4 +bevacuzimab Arm 3: XELOX + bevacuzimab	3-year DFS 5-year OS	Median follow-up 60 months
NSABBP C-08	Allegra et al. 2013 [31]	2672	Stage II-III colon cancer patients, Sept 2004 –Oct 2006	Arm 1: Modified FOLFOX6 Arm 2: modified FOLFOX6 + bevacizumab	3-year DFS	Median follow-up 5 years
Alliance	Meyerhardt et al. 2021 [33]	2526	Stage III Colon cancer patients, June 2010 – Nov 2015	Arm 1: 3 months FOLFOX + celecoxib Arm 2: 6 months FOLFOX + celecoxib Arm 3: 3 months FOLFOX + placebo Arm 4: 6 month FOLFOX + placebo	3-year DFS 5-year OS	Median follow-up 6 years
Currently ongoing						
Study Identifier (Acronym)	**Author**	**Target no. of patients**	**Enrolment**	**Interventions**	**Outcome**	
PRODIGE 50-ASPIK	Michel et al. 2018 [36]	264	High risk stage II and stage III colon cancer patients with PIK3CA mutation	Arm 1: Aspirin Arm 2: Placebo	DFS	
ASCOLT	Ali et al. 2011 [37]	1587	High risk stage II and stage III colon cancer patients	Arm 1: Aspirin Arm 2: Placebo	DFS	
EPISODE-III	Takashima et al. 2019 [38]	880	Stage III colon cancer patients	Arm 1: Aspirin Arm 2: Placebo	DFS	
ASPIRIN trial		1588	Stage II and stage III colon cancer patients	Arm 1: Aspirin Arm 2: Placebo	DFS	

Abbreviations: FOLFOX: leucovorin (folinic acid) & fluorouracil & oxaliplatin, CAPOX: capecitabine & oxaliplatin, XELOX: capecitabine & oxaliplatin

tor (celecoxib) versus placebo [33]. Results showed that the addition of celecoxib for 3 years, compared with placebo, to standard adjuvant chemotherapy did not significantly improve 3-year DFS or 5-year OS.

Based on two retrospective studies showing that low-dose aspirin as a targeted adjuvant therapy can improve DFS in colon cancer patients with mutated PIK3CA tumors, the PRODIGE 50-ASPIK trial is currently being performed [34-36]. In this trial, patients with radically resected high risk stage II (MSS) or stage III colon cancer with PIK3CA mutation are randomized between adjuvant treatment (CAPOX, FOLFOX, Capecitabine, 5-FU/LV) with acetylsalicylic acid (aspirin) versus placebo, with DFS as the primary endpoint. Similarly, there are three other ongoing placebo controlled randomized trials investigating the effect of adding aspirin to adjuvant chemotherapy in stage II-III colon cancer patients [37-39].

Table 4. Studies on immunotherapy

Study Identifier (Acronym)	Author	No. of patients	Enrolment	Interventions	Outcome	Length of follow-up
			Completed trials			
NICHE	Chalabi et al. [90]	40	Colon cancer patients with dMMR or pMMR tumors, march 2017 – ongoing	Arm 1: ipilimumab/ nivolumab Arm 2: ipilimumab/ nivolumab with celecoxib	Safety and feasibility	Median follow-up 9 months
			Currently ongoing			
Study Identifier (Acronym)	**Author**	**Target no. of patients**	**Enrolement**	**Interventions**	**Outcome**	
POLEM	Lau et al. 2020 [42]	402	Stage III colon cancer patients with a Locally confirmed dMMR tumor	Arm 1: standard fluoropyrimidine-based adjuvant chemotherapy Arm 2: fluoropyrimidine based adjuvant chemotherapy followed by Avelumab	DFS	
ATOMIC	Frank et al. 2019 [43]	700	Stage III colon cancer patients with evidence of dMMR	Arm 1: mFOLFOX6 Arm 2: mFOLFOX6 combined with atezolizumab	DFS	

Abbreviations: dMMR: deficient mismatch repair, pMMR: proficient mismatch repair, FOLFOX: leucovorin calcium (folinic acid) & fluorouracil & oxaliplatin

Immunotherapy (Table 4)

As stated before, the prognosis of colon cancer patients with a dMMR tumor is much better compared with patients with pMMR tumors in early stage disease. Plus, dMMR is predictive for lack of response to fluoropyrimidine-monotherapy [12, 13]. Therefore, the ESMO guidelines recommend to refrain from adjuvant chemotherapy in high-risk stage II dMMR colon cancer patients as the possible clinical benefit is too low [40]. In the exploratory NICHE study, patients with stage I-III resectable colon cancer were included. Patients with dMMR or pMMR tumors received a single dose of ipilimumab and two doses of nivolumab in neoadjuvant setting and the pMMR group with or without celecoxib. Results showed that treatment was well tolerated and the primary endpoint of radical resection without delay for all included patients, was met. Pathological response was observed in all dMMR tumors and in 27% of pMMR tumors. This data needs to be validated and DFS results have to be awaited [41]. Furthermore, two other RCTs are currently being performed in dMMR-patients. The POLEM study aims to determine if the addition of the anti-PD-L1 antibody avelumab following adjuvant chemotherapy improves DFS in patients with stage III dMMR or POLE mutant colon cancer [42]. The ATOMIC trial aims to determine if the addition of anti-PD-L1 antibody atezolizumab to adjuvant FOLFOX versus FOLFOX alone can improve DFS in patients with stage III dMMR colon cancer [43].

Duration of chemotherapy (Table 5)

For years, adjuvant treatment for patients with stage III colon cancer comprised of 6 months of fluoropyrimidine and oxaliplatin-based adjuvant chemotherapy. Recently, a pooled non-inferiority analysis of six randomized trials comparing 3 to 6 months of adjuvant FOLFOX or CAPOX chemotherapy in a total of 12835 stage III colon cancer patients was published (IDEA trial) [9]. The exact 5-year DFS and 5-year OS results are listed in Table 5. Results did not meet the primary endpoint of noninferiority regarding DFS and OS for the whole population, however, an unexpected significant interaction effect of the regimen used (FOLFOX vs CAPOX) was detected in a pre-planned analysis. Treatment with 3 months of CAPOX was non-inferior to 6 months regarding 5-year DFS and 5-year OS. For treatment with FOLFOX, treatment with 6 months of therapy was superior to 3 months regarding both 5-year DFS and OS. In patients classified as low risk (pathological stage T1–3 N1), 3 months was non-inferior to 6 months regarding 5-year DFS and 5-year OS. This was not reflected in the higher risk category (pathological T4 or N2), where 6 months was superior to 3 months in both 5-year DFS and 5-year OS.

Based on these subgroup results from the IDEA study the aforementioned IROCAS-study has been conducted, to analyze whether the addition of irinotecan to FOLFOX (FOLFIRINOX), improves DFS of high risk stage III patients [26].

Table 5. Duration

Study Identifier (Acronym)	Author	No. of patients	Enrolment	Interventions	Outcome	Length of follow-up
IDEA	Andre et al. 2020 [91]	12835	Stage III colon cancer patients, June 2007 - Dec 2015	FOLFOX or CAPOX administered for 3 months, versus 6 months.	5-year DFS 5-year OS	Median follow-up 72.3 months

	Subgroup analyses									
	Overall		**CAPOX**		**FOLFOX**		**Low risk**		**High risk**	
	N = 12835		N = 5064 (39.5%)		N = 7771 (60.5%)		N= 7507 (58.5%)		N= 5273 (41.1%)	
	3 months	6 months	3 months	6 months	3 months	6 months	3 months	6 months	3 months	6 months
5-year DFS										
HR (95% CI)	1.08 (1.02–1.15)		0.98 (0.88–1.08)		1.16 (1.07–1.26)		1·04 (0·94–1·15)		1·13 (1·03–1·22)	
Non-inferiority P-value	0.25		0.027		0.80		0.16		0.63	
5-year OS										
5-year OS, % (95% CI)	82.4 (81.4 — 83.3)	82.8 (81.8 – 83.8)	82.1 (80.5 –83.6)	81.3 (79.2 – 82.9)	82.6 (81.3 – 83.8)	83.8 (82.6 – 85.0)	89.6 (88.6 –90.7)	88.9 (79.2 – 82.9)	82.6 (81.3 – 83.8)	83.8 (82.6 – 85.0)
Δ5-year OS, %	–0.4		+0.9		-1.2		+0.7		-2.1	
HR (95% CI)	1.02 (0.95—1.12)		0.96 (0.85–1.08)		1.07 (0.97–1.18)		1·04 (0·94–1·15)		1·13 (1·03–1·22)	
Non-inferiority P-value	–0.4		+0.9		–1.2		0.16		0.63	

Abbreviations: FOLFOX: leucovorin calcium (folinic acid) & fluorouracil & oxaliplatin, CAPOX: capecitabine & oxaliplatin

Neoadjuvant Chemotherapy (Table 6)

Neoadjuvant chemotherapy can have several advantages regarding oncological outcomes: the possibility of response monitoring, early eradication of micrometastases and more complete resections. Neoadjuvant treatment is already standard of care for different gastrointestinal malignancies including esophageal, gastric and rectal cancers with significant benefitson DFS and OS [44-48]. The FOxTROT Collaborative Group was the first to set up a neoadjuvant trial in colon cancer patients to evaluate the effect of neoadjuvant chemotherapy with FOLFOX or CAPOX [49]. Patients with a cT3-4N0-2M0 colon cancer were randomized 2:1 between 6 weeks of neoadjuvant combined with 18 weeks of adjuvant FOLFOX/CAPOX or 24 weeks of adjuvant FOLFOX/CAPOX. The primary endpoint, decreasing the 2-year recurrence rate, was not significant. However, a strong tendency in reducing the risk of 2-year recurrence rate was seen with 13.6% recurrence in the perioperative chemotherapy group compared to 17.2% in the group treated with adjuvant chemotherapy alone [50]. Furthermore, serious perioperative morbidity was lower in the neoadjuvant chemotherapy arm and significantly more complete resection margins were achieved compared with the adjuvant chemotherapy arm. Importantly, the pathological tumor response was evidently associated with recurrence free survival. Patients with a complete response developed no recurrences after 2 years of follow-up, compared to 26% of patients that showed no regression at all [51]. This illustrates that the response to chemotherapy of the primary tumor may indeed be a reliable measurement for chemotherapy efficiency. A follow-up trial on the FOXTROT study is currently initiated within an international collaborative network.

Table 6. Neoadjuvant chemotherapy

Study Identifier (Acronym)	Author	No. of patients	Enrolment	Interventions	Outcome	Length of follow-up
Completed trials						
FOXTROT	Seymour et al. [50]	1052	High risk stage II- Stage III colon cancer, june 2008-Dec 2016	Arm 1: 6 weeks of neoadjuvant combined with 18 weeks of adjuvant FOLFOX/CAPOX Arm 2: 24 weeks of adjuvant FOLFOX/CAPOX.	DFS	2 years

Abbreviations: FOLFOX: leucovorin calcium (folinic acid) & fluorouracil & oxaliplatin, CAPOX: capecitabine & oxaliplatin

Circulating tumor DNA (Table 7)

In attempts to enhance patient selection for systemic treatment, new prognostic and predictive biomarkers are studied which led to the discovery of circulating tumor DNA (ctDNA).

Table 7. Currently ongoing RCTs on ctDNA

Study Identifier (Acronym)	Target no. of patients	Enrolement	Interventions	Outcome
COBRA[92]	1408	Resected stage IIA colon cancer patients without high-risk features	Arm 1: Active surveillance. Arm 2: ctDNA directed therapy (ctDNA positive→ adjuvant chemotherapy, ctDNA negative→active surveillance).	Clearance of ctDNA with ACT (phase II) and RFS (phase III)
NCT03803553	500	Resected stage III colon cancer patients	Enrolment after standard adjuvant chemotherapy in one of the 2 arms: Arm 1. ctDNA negative: Surveillance; Arm 2. ctDNA positive: (a) MSS patients→6 months of FOLFIRI vs. surveillance, (b) MSI high→6 months of nivolumab, (c) BRAF mutant/MSS→6 months of BRAF directed therapy.	5-year DFS and clearance rate of ctDNA.
DYNAMIC-III	1000	Resected stage III colon cancer patients	Arm 1: Standard of care. Arm 2: ctDNA informed (ctDNA negative→therapy de-escalation; ctDNA positive→therapy escalation).	3-year RFS
DYNAMIC	450	Resected stage II colon cancer patients	Arm 1: positive ctDNA→Adjuvant chemotherapy, negative ctDNA→surveillance. Arm 2: Treated at the discretion of the clinicians.	3-year RFS
MEDOCC-CREATE [93]	1320	Resected stage II colon cancer patients	Arm 1: standard of care. Arm 2: ctDNA directed therapy (ctDNA positive→ CAPOX for 6 months, ctDNA negative→active surveillance).	Fraction of patients receiving ACT when ctDNA is detectable after resection. 2-year recurrence rate
TRACC [94]	1621	High risk stage II, stage III colorectal cancer patients	Arm 1: 6 months of capecitabine or 3 months of CAPOX Arm 2: ctDNA-guided: ctDNA-positive → standard adjuvant chemotherapy; ctDNA-negative → de-escalate treatment but re-escalate if ctDNA becomes positive at 3 months	Non-inferiority in 3-year DFS between standard of care arm and ctDNA-guided adjuvant chemotherapy arm
CIRCULATE PRODEGY [95]	198	Stage II colon cancer patients	ctDNA-positive patients randomised to: Arm 1: surveillance Arm 2: adjuvant FOLFOX	To demonstrate a 17.5% gain in 3-year DFS in post-op ctDNA-positive patients treated with adjuvant FOLFOX compared to observation alone

Abbreviations: FOLFOX: leucovorin calcium (folinic acid) & fluorouracil & oxaliplatin, CAPOX: capecitabine & oxaliplatin, FOLFORI: 5-FU/LV + irinotecan

ctDNA can be found in the blood of cancer patients as a result of neoplastic cell necrosis and DNA release. The detection of ctDNA in the plasma of colon cancer patients (known as liquid biopsies) has been successfully used to monitor tumor burden and therapy resistance, to evaluate the presence of residual disease after potentially curative treatment and to monitor disease recurrence with high sensitivity and specificity [52-54]. However, implementation of ctDNA in clinical practice is still hampered by the lack of standardized methods for ctDNA processing and analysis. Plus, it remains unclear if ctDNA positive patients are also the patients in which adjuvant chemotherapy can prevent recurrence and if adjuvant chemotherapy can be withheld in ctDNA negative patients. Therefore, multiple RCTs all over the world are momentarily being performed on the prognostic and predictive role of ctDNA in early stage colon cancer. Results are expected to have a large impact on precision treatment with hopefully less overtreatment and undertreatment for colon cancer patients.

DISCUSSION

The only adjuvant regimens with a validated effect on long-term clinical outcome in stage III colon cancer patients are the oxaliplatin-based chemotherapy regimens FOLFOX or CAPOX when patients are eligible to a doublet treatment. Multiple RCTs studying different monotherapy schedules of fluoropyrimidines showed equivalent effect in stage III patients [14, 16, 22, 23]. These results are in line with previous literature [3, 6-8]. Adjuvant chemotherapy has since been implemented as standard of care. For high risk stage II colon cancer, the ten-year results from the MOSAIC trial showed no overall survival benefit for patients who received adjuvant chemotherapy (18). However, other studies were contradictory or inconclusive [55, 56]. As mentioned before, eligibility of patients for adjuvant treatment has changed, mostly as a result of altered definitions of high-risk stage II disease. It is therefore important to note that most of the high risk features used in these studies have been abandoned based on recent studies showing that pT4 is the only positive predictive factor for a significant benefit of adjuvant chemotherapy in stage II colon cancer [57, 58]. Based on these non-randomized, mostly retrospective cohort studies, pT4 is currently the only remaining indication for adjuvant chemotherapy in stage II colon cancer in different international guidelines [59, 60].

The most significant progress made in systemic treatment over the past decade is reducing the duration of adjuvant chemotherapy from 6 to 3 months in stage III colon cancer patients. The IDEA trial showed that chemotherapy de-escalation is safe in terms of long-term outcome resulting in a significant reduction in toxicity [9]. A questionnaire study showed that the IDEA has shifted clinician preference from FOLFOX to CAPOX for adjuvant therapy, and that most clinicians now use a risk-stratified approach in determining duration of adjuvant therapy [61]. The updated 5-year survival results were published and the investigators concluded: “Noninferior-

ity of 3 months vs 6 months of adjuvant chemotherapy for patients with stage III colon cancer was not confirmed in terms of overall survival, but the absolute 0.4% difference in 5-year overall survival should be placed in clinical context. Overall survival results support the use of 3 months of adjuvant CAPOX for most patients with stage III colon cancer. This conclusion is strengthened by the substantial reduction of toxicities, inconveniencies, and cost associated with a shorter treatment duration." [9]

In addition to the duration of chemotherapy, the timing of systemic therapy is also a topical issue. Results of the first phase III RCT on neoadjuvant chemotherapy are very promising and are likely to have a big impact on treatment strategy for a large group of high risk stage II/ stage III colon cancer patients [50]. Besides the FOXTROT study, the PRODIGE 22 phase 2 RCT also showed that neoadjuvant chemotherapy was well tolerated with no increase in surgical morbidity [62]. Importantly, a neoadjuvant approach requires reliable clinical TNM staging to minimize the risk of overtreating patients with stage I or low risk stage II colon cancer. A meta-analysis analyzing the accuracy of T and N staging on CT imaging showed that T1-2 can be reliably distinguished from T3-4 (sensitivity 96% and specificity 70%), while nodal involvement is unreliable with a pooled sensitivity and specificity of 78% and 68% respectively [63]. In the FOXTROT-study and PRODIGE 22, similar results were found [50, 62]. Approximately 25% of patients were erroneously staged and consequently treated with chemotherapy who would otherwise not qualify for adjuvant chemotherapy according to current standard of care. These results underline the need for improving pre-treatment disease assessment in this setting. A recent study evaluated whether staging of colon cancer on CT can be improved by training radiology residents [64]. Results show that the diagnostic performance of T staging of colon cancer on CT-scan improved significantly after analyzing multiple scans. However, N staging on CT did not show a significant improvement over time. Feedback from an expert in between scans did not lead to an improvement in performance suggesting that experience itself would be sufficient for radiologists to become accurate. This still leaves the need for accurate N staging. Exploratory data suggests a possible role for MRI or PET in preoperative staging [65, 66]. However, these techniques have not been validated in randomized trials and data is limited to small case series. More refinement of imaging techniques are needed to optimize patient selection for neoadjuvant treatment.

It is demonstrated that dMMR is favorable prognostic marker in stage II colon cancer and data from stage II-III colon cancer patients treated with 5-FU-based adjuvant therapy versus observation have confirmed a lack of benefit for 5-FU in dMMR tumors [12]. However, a recent pooled analyses of 12 adjuvant trials showed that adding oxaliplatin to fluopyrimidine improves OS and DFS in stage III dMMR patients [67]. Furthermore, results showed that compared with pMMR, dMMR patients experienced better outcomes in the N1 group but similar survival in the N2 group [67]. It is apparent that patient stratification based on MMR status is

receiving increasing attention. In particular with the impressive results of the NICHE-study [41]. Subgroup analyses from the CALGB 89803 study, showed improved 5-year DFS results in dMMR patients treated with IFL as compared with those receiving 5-FU/LV [68]. However, this benefit from irinotecan in dMMR patients was not confirmed in subgroup analyses from the PETACC-3 study [69]. A pooled analysis on the prognostic value of BRAF and KRAS mutations within dMMR and pMMR subgroups of resected colon cancer patients treated with FOLFOX, showed that BRAF or KRAS mutations are independently associated with worse survival outcomes in patients with pMMR, but not dMMR tumors [70]. Interestingly, analyses form the NSABP-C08 trial showed that patients diagnosed with dMMR tumors had a significant survival benefit from the addition of bevacizumab while no benefit was observed in patients diagnosed with pMMR tumors [32]. Although this was a post hoc analysis, this data suggests that a molecularly defined subset of colon cancer patients may have clinical benefit from targeted agents and underscores the need for independent validation in other clinical trials. Recently, results were published from the KEYNOTE-177 study [71]. In this RCT, the efficacy and safety of pembrolizumab versus standard of care chemotherapy ± bevacizumab or cetuximab was analyzed, as first-line therapy for patients with dMMR metastatic CRC patients. Treatment with pembrolizumab led to significantly longer progression-free survival than chemotherapy with fewer treatment-related adverse events. These results, along with the first results from the NICHE-study, support ongoing trials investigating immune checkpoint inhibitors, such as the ongoing ATOMIC trial involving patients with dMMR stage III colon cancer, in which the anti–PD-L1 antibody atezolizumab is added to adjuvant FOLFOX [41, 72]. Other current ongoing trials on treatment for patients with dMMR and pMMR tumors, PIK-3CA mutations, and POLE mutations, are eagerly awaited [41-43]. Furthermore, treatment with anti-PDL1 monoclonal antibodies and treatment with aspirin or celecoxib combined with adjuvant chemotherapy may provide additional benefit in the risk of recurrence in patients with high-risk stage II or stage III tumors [73].

As previously mentioned, despite many efforts on improving systemic treatment in high-risk stage II and stage III patients, it is currently not possible to accurately predict and identify the patients who will develop recurrence without adjuvant therapy which hampers optimal patient selection. With the introduction of ctDNA, patients with a high risk of recurrence can be identified and several studies have reported that serial monitoring of ctDNA can also provide valuable information on the efficacy of adjuvant chemotherapy [74-76]. However, a high risk of recurrence is not necessarily solved by administering more or heavier chemotherapy regimens. More and more, translational research suggests that response to chemotherapy is strongly related to tumor biology. Therefore, risk of recurrence may be predicted based on tumor response to chemotherapy. The Consensus Molecular Subtypes (CMSs) have been developed to stratify colon cancer into biologically and clinically distinct subtypes [77]. Besides the prognostic value, CMS seems to have a predictive value as well for response to systemic treatment [78, 79]. In the

CONNECTION-study, a prospective, single arm, multicenter intervention study within PLCRC, including patients with resectable MSS cT3-4NxM0 colon cancer, patients are treated with neoadjuvant CAPOX to evaluate CMS as a possible predictive biomarker for response to (neo-adjuvant) chemotherapy [80]. Furthermore, ctDNA-analyses will be performed to monitor treatment response to neoadjuvant treatment and detect residual disease. This study can provide the results necessary to proceed to future studies in which (neo)adjuvant chemotherapy may be withheld in patients with a specific CMS subtype, who show no benefit from chemotherapy and for whom possible new treatments can be investigated. Besides CMS, another promising prognostic stratification in colon cancer patients is by means of tumor-infiltrating lymphocytes (TILs). Tumor-infiltrating lymphocyte densities have shown prognostic significance in well-defined cohorts of patients with stage II and III colon cancer, and the developed Immunoscore has been externally validated [81-84]. Analysis of TILs could enable more selective use of adjuvant therapy in both stage II and stage III patients [85]. An important, yet unanswered, question is the possible correlation between TILs and ctDNA. Some results suggest a predictive potential of TILs for chemotherapy outcomes but future studies are warranted to analyze the predictive value of TILs and their correlation with ctDNA [84, 86].

In conclusion, the only significant progress made in systemic treatment over the past 10 years is reducing the duration of adjuvant chemotherapy from 6 to 3 months in stage III patients. None of the RCTs on new agents showed survival benefit over conventional therapies. Defining the utility of information gathered from prognostic and predictive molecular biomarkers for clinical management of colon cancer patients is warranted. Even though knowledge about prognosis and prediction has improved by molecular analyses, validation of the added value of biomarkers in RCTs is lacking which hampers implementation in daily practice. It is essential that promising developments such as neoadjuvant treatment, immunotherapy for MSI and ctDNA are studied in RCTs followed by validation to be able to implement these in daily practice. The increasing number of subgroups in colon cancer results in smaller patient numbers for specific clinical trials. This stresses the need for international collaborations such as the IDEA collaboration and the proposed FOxTROT collaboration.

REFERENCES

1. Ferlay J EM, Lam F, Colombet M, Mery L, Piñeros M, et al. Global Cancer Observatory: Cancer Today. Lyon: International Agency for Research on Cancer; 2020. 2020.
2. Brouwer NPM, Bos A, Lemmens V, et al. An overview of 25 years of incidence, treatment and outcome of colorectal cancer patients. *Int J Cancer* 2018; 143(11):2758-2766.
3. André T, Boni C, Navarro M, et al. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. *J Clin Oncol* 2009; 27(19):3109-3116.
4. Haller DG, Tabernero J, Maroun J, et al. Capecitabine plus oxaliplatin compared with fluorouracil and folinic acid as adjuvant therapy for stage III colon cancer. *J Clin Oncol* 2011; 29(11):1465-1471.
5. Moertel CG, Fleming TR, Macdonald JS, et al. Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. *N Engl J Med* 1990; 322(6):352-8.
6. Kuebler JP, Wieand HS, O'Connell MJ, et al. Oxaliplatin combined with weekly bolus fluorouracil and leucovorin as surgical adjuvant chemotherapy for stage II and III colon cancer: results from NSABP C-07. *J Clin Oncol* 2007; 25(16):2198-204.
7. Yothers G, O'Connell MJ, Allegra CJ, et al. Oxaliplatin as adjuvant therapy for colon cancer: updated results of NSABP C-07 trial, including survival and subset analyses. *J Clin Oncol* 2011; 29(28):3768-74.
8. Andre T, Boni C, Mounedji-Boudiaf L, et al. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. *N Engl J Med* 2004; 350(23):2343-51.
9. Andre T, Meyerhardt J, Iveson T, et al. Effect of duration of adjuvant chemotherapy for patients with stage III colon cancer (IDEA collaboration): final results from a prospective, pooled analysis of six randomised, phase 3 trials. *Lancet Oncol* 2020; 21(12):1620-1629.
10. Moertel CG, Fleming TR, Macdonald JS, et al. Fluorouracil plus levamisole as effective adjuvant therapy after resection of stage III colon carcinoma: a final report. *Ann Intern Med* 1995; 122(5):321-6.
11. Elferink MA, de Jong KP, Klaase JM, et al. Metachronous metastases from colorectal cancer: a population-based study in North-East Netherlands. *Int J Colorectal Dis* 2015; 30(2):205-12.
12. Sargent DJ, Marsoni S, Monges G, et al. Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. *J Clin Oncol* 2010; 28(20):3219-26.
13. Des Guetz G, Schischmanoff O, Nicolas P, et al. Does microsatellite instability predict the efficacy of adjuvant chemotherapy in colorectal cancer? A systematic review with meta-analysis. *Eur J Cancer* 2009; 45(10):1890-6.
14. Twelves C, Scheithauer W, McKendrick J, et al. Capecitabine versus 5-fluorouracil/folinic acid as adjuvant therapy for stage III colon cancer: Final results from the X-ACT trial with analysis by age and preliminary evidence of a pharmacodynamic marker of efficacy. *Ann Oncol* 2012; 23(5):1190-1197.
15. Shimada Y, Hamaguchi T, Mizusawa J, et al. Randomised phase III trial of adjuvant chemotherapy with oral uracil and tegafur plus leucovorin versus intravenous fluorouracil and levofolinate in patients with stage III colorectal cancer who have undergone Japanese D2/D3 lymph node dissection: Final results of JCOG0205. *Eur J Cancer* 2014; 50(13):2231-2240.
16. Pectasides D, Karavasilis V, Papaxoinis G, et al. Randomized phase III clinical trial comparing the combination of capecitabine and oxaliplatin (CAPOX) with the combination of 5-fluoro-

uracil, leucovorin and oxaliplatin (modified FOLFOX6) as adjuvant therapy in patients with operated high-risk stage II or stage III colorectal cancer. *BMC Cancer* 2015; 15(1).

17. Hong YS, Park YS, Lim HY, et al. S-1 plus oxaliplatin versus capecitabine plus oxaliplatin for first-line treatment of patients with metastatic colorectal cancer: a randomised, non-inferiority phase 3 trial. *Lancet Oncol* 2012; 13(11):1125-32.
18. Yamada Y, Takahari D, Matsumoto H, et al. Leucovorin, fluorouracil, and oxaliplatin plus bevacizumab versus S-1 and oxaliplatin plus bevacizumab in patients with metastatic colorectal cancer (SOFT): an open-label, non-inferiority, randomised phase 3 trial. *Lancet Oncol* 2013; 14(13):1278-86.
19. Kusumoto T, Ishiguro M, Nakatani E, et al. Updated 5-year survival and exploratory T x N subset analyses of ACTS-CC trial: A randomised controlled trial of S-1 versus tegafur-uracil/ leucovorin as adjuvant chemotherapy for stage III colon cancer. *ESMO Open* 2018; 3(6).
20. Sunami E, Kusumoto T, Ota M, et al. S-1 and Oxaliplatin Versus Tegafur-uracil and Leucovorin as Postoperative Adjuvant Chemotherapy in Patients With High-risk Stage III Colon Cancer (ACTS-CC 02): A Randomized, Open-label, Multicenter, Phase III Superiority Trial. *Clin Colorectal Cancer* 2020.
21. Hamaguchi T, Shimada Y, Mizusawa J, et al. Randomized phase III study of adjuvant chemotherapy with S-1 versus capecitabine in patients with stage III colorectal cancer: Updated results of Japan Clinical Oncology Group study (JCOG0910). *Ann Oncol* 2017; 28:v163.
22. Andre T, Boni C, Navarro M, et al. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. *J Clin Oncol* 2009; 27(19):3109-16.
23. André T, De Gramont A, Vernerey D, et al. Adjuvant fluorouracil, leucovorin, and oxaliplatin in stage II to III colon cancer: Updated 10-year survival and outcomes according to BRAF mutation and mismatch repair status of the MOSAIC study. *J Clin Oncol* 2015; 33(35):4176-4187.
24. Papadimitriou CA, Papakostas P, Karina M, et al. A randomized phase III trial of adjuvant chemotherapy with irinotecan, leucovorin and fluorouracil versus leucovorin and fluorouracil for stage II and III colon cancer: A Hellenic Cooperative Oncology Group study. *BMC Med* 2011; 9.
25. Paschke S, Hebart H, Goeb R, et al. Adjuvant chemotherapy of locally advanced colon cancer: Final results of a randomized trial comparing 5-fluorouracil and folinic acid with folfiri. *Visc Med* 2019; 35(2):124-132.
26. Bennouna J, Andre T, Miglianico L, et al. PRODIGE 52-UCGI 29-CCTG/CO.27 (IROCAS): A multicenter, international, randomized phase III trial comparing adjuvant modified (m) FOLFIRINOX to mFOLFOX6 in patients with high-risk stage III (pT4 and/or N2) colon cancer (a UNICANCER GI-PRODIGE trial). *J Clin Oncol* 2018; 36(15).
27. Alberts SR, Sargent DJ, Nair S, et al. Effect of oxaliplatin, fluorouracil, and leucovorin with or without cetuximab on survival among patients with resected stage III colon cancer: A randomized trial. *J Am Med Assoc* 2012; 307(13):1383-1393.
28. Taieb J, Tabernero J, Mini E, et al. Oxaliplatin, fluorouracil, and leucovorin with or without cetuximab in patients with resected stage III colon cancer (PETACC-8): An open-label, randomised phase 3 trial. *Lancet Oncol* 2014; 15(8):862-873.
29. De Gramont A, Henriques J, Baruch B, et al. Bevacizumab plus oxaliplatin-based chemotherapy as adjuvant treatment for colon cancer (CC): Updated analysis of stage II disease from the AVANT phase III randomized trial by the GERCOR group. *Ann Oncol* 2019; 30:v209.

30. de Gramont A, Van Cutsem E, Schmoll HJ, et al. Bevacizumab plus oxaliplatin-based chemotherapy as adjuvant treatment for colon cancer (AVANT): A phase 3 randomised controlled trial. *Lancet Oncol* 2012; 13(12):1225-1233.
31. Allegra CJ, Yothers G, O'Connell MJ, et al. Bevacizumab in stage II-III colon cancer: 5-year update of the National Surgical Adjuvant Breast and Bowel Project C-08 trial. *J Clin Oncol* 2013; 31(3):359-364.
32. Pogue-Geile K, Yothers G, Taniyama Y, et al. Defective mismatch repair and benefit from bevacizumab for colon cancer: findings from NSABP C-08. *J Natl Cancer Inst* 2013; 105(13):989-92.
33. Meyerhardt JA, Shi Q, Fuchs CS, et al. Effect of Celecoxib vs Placebo Added to Standard Adjuvant Therapy on Disease-Free Survival Among Patients With Stage III Colon Cancer: The CALGB/SWOG 80702 (Alliance) Randomized Clinical Trial. *JAMA* 2021; 325(13):1277-1286.
34. Liao X, Lochhead P, Nishihara R, et al. Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. *N Engl J Med* 2012; 367(17):1596-606.
35. Domingo E, Church DN, Sieber O, et al. Evaluation of PIK3CA mutation as a predictor of benefit from nonsteroidal anti-inflammatory drug therapy in colorectal cancer. *J Clin Oncol* 2013; 31(34):4297-305.
36. Michel P, Boige V, Andre T, et al. Aspirin versus placebo in stage III or high-risk stage II colon cancer with PIK3CA mutation: A French randomised double-blind phase III trial (PRODIGE 50-ASPIK). *Dig Liver Dis* 2018; 50(3):305-307.
37. Ali R, Toh HC, Chia WK. The utility of Aspirin in dukes C and high risk dukes B colorectal cancer - The ASCOLT study: Study protocol for a randomized controlled trial. *Trials* 2011; 12.
38. Takashima A, Miyamoto K, Sato Y, et al. Efficacy of aspirin for stage III colorectal cancer: a randomized double-blind placebocontrolled trial (JCOG1503C, EPISODE-III trial). *Journal of clinical oncology* 2019; Conference: 2019 Annual Meeting of the American Society of Clinical Oncology, ASCO 2019. United States. 37(Supplement 15).
39. https://clinicaltrials.gov/ct2/show/NCT02301286.
40. Committee EG. eUpdate - Early Colon Cancer Treatment Recommendations. 2019.
41. Chalabi M, Fanchi LF, Dijkstra KK, et al. Neoadjuvant immunotherapy leads to pathological responses in MMR-proficient and MMR-deficient early-stage colon cancers. *Nature Medicine* 2020; 26(4):566-576.
42. Lau D, Kalaitzaki E, Church DN, et al. Rationale and design of the POLEM trial: avelumab plus fluoropyrimidine-based chemotherapy as adjuvant treatment for stage III mismatch repair deficient or POLE exonuclease domain mutant colon cancer: a phase III randomised study. *ESMO Open* 2020; 5(1).
43. Frank AS, Fang-Shu O, Tyler Z, et al. Randomized trial of standard chemotherapy alone or combined with atezolizumab as adjuvant therapy for patients with stage III colon cancer and deficient mismatch repair (ATOMIC, Alliance A021502). *Journal of Clinical Oncology* 2019; 37(15_suppl):e15169-e15169.
44. van Hagen P, Hulshof MC, van Lanschot JJ, et al. Preoperative chemoradiotherapy for esophageal or junctional cancer. *N Engl J Med* 2012; 366(22):2074-84.
45. Bosset JF, Mercier M, Triboulet JP, et al. Surgical resection with and without chemotherapy in oesophageal cancer. *Lancet* 2002; 360(9340):1173-4; author reply 1175.
46. Cunningham D, Allum WH, Stenning SP, et al. Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. *N Engl J Med* 2006; 355(1):11-20.

47. Sebag-Montefiore D, Stephens RJ, Steele R, et al. Preoperative radiotherapy versus selective postoperative chemoradiotherapy in patients with rectal cancer (MRC CR07 and NCIC-CTG C016): a multicentre, randomised trial. *Lancet* 2009; 373(9666):811-20.
48. Roh MS, Yothers GA, Connell MJO, et al. The impact of capecitabine and oxaliplatin in the preoperative multimodality treatment in patients with carcinoma of the rectum: NSABP R-04. *Journal of Clinical Oncology* 2011; 29(15_suppl):3503-3503.
49. Foxtrot Collaborative G. Feasibility of preoperative chemotherapy for locally advanced, operable colon cancer: the pilot phase of a randomised controlled trial. *Lancet Oncol* 2012; 13(11):1152-60.
50. Matthew TS, Dion M, on behalf of the International FTI. FOxTROT: an international randomised controlled trial in 1052 patients (pts) evaluating neoadjuvant chemotherapy (NAC) for colon cancer. *Journal of Clinical Oncology* 2019; 37(15_suppl):3504-3504.
51. Matthew T. Seymour DM, and on behalf of the International FOxTROT Trial Investigators. FOxTROT: an international randomised controlled trial in 1052 patients (pts) evaluating neo-adjuvant chemotherapy (NAC) for colon cancer. Journal of Clinical Oncology 2019.
52. Crowley E, Di Nicolantonio F, Loupakis F, et al. Liquid biopsy: monitoring cancer-genetics in the blood. *Nat Rev Clin Oncol* 2013; 10(8):472-84.
53. Diaz LA, Jr., Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. *J Clin Oncol* 2014; 32(6):579-86.
54. Tie J, Cohen JD, Wang Y, et al. Circulating Tumor DNA Analyses as Markers of Recurrence Risk and Benefit of Adjuvant Therapy for Stage III Colon Cancer. *JAMA Oncol* 2019.
55. Benson AB, 3rd, Schrag D, Somerfield MR, et al. American Society of Clinical Oncology recommendations on adjuvant chemotherapy for stage II colon cancer. *J Clin Oncol* 2004; 22(16):3408-19.
56. Labianca R, Nordlinger B, Beretta GD, et al. Early colon cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2013; 24 Suppl 6:vi64-72.
57. Verhoeff SR, van Erning FN, Lemmens VE, et al. Adjuvant chemotherapy is not associated with improved survival for all high-risk factors in stage II colon cancer. *Int J Cancer* 2016; 139(1):187-93.
58. Babcock BD, Aljehani MA, Jabo B, et al. High-Risk Stage II Colon Cancer: Not All Risks Are Created Equal. *Ann Surg Oncol* 2018; 25(7):1980-1985.
59. Costas-Chavarri A, Nandakumar G, Temin S, et al. Treatment of Patients With Early-Stage Colorectal Cancer: ASCO Resource-Stratified Guideline. *J Glob Oncol* 2019; 5:1-19.
60. Argiles G, Tabernero J, Labianca R, et al. Localised colon cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2020; 31(10):1291-1305.
61. Yu IS, Pereira AAL, Lee M, et al. Medical Oncologists' Perspectives on How the Results of the IDEA Collaboration Impact the Adjuvant Treatment of Stage III Colon Cancer. *The Oncologist* 2020; 25(3):229-234.
62. Karoui M, Rullier A, Piessen G, et al. Perioperative FOLFOX 4 Versus FOLFOX 4 Plus Cetuximab Versus Immediate Surgery for High-Risk Stage II and III Colon Cancers: A Phase II Multicenter Randomized Controlled Trial (PRODIGE 22). *Ann Surg* 2020; 271(4):637-645.
63. Nerad E, Lahaye MJ, Maas M, et al. Diagnostic Accuracy of CT for Local Staging of Colon Cancer: A Systematic Review and Meta-Analysis. *AJR Am J Roentgenol* 2016; 207(5):984-995.
64. Hong EK, Castagnoli F, Gennaro N, et al. Locoregional CT staging of colon cancer: does a learning curve exist? *Abdom Radiol (NY)* 2021; 46(2):476-485.

65. Tsunoda Y, Ito M, Fujii H, et al. Preoperative diagnosis of lymph node metastases of colorectal cancer by FDG-PET/CT. *Jpn J Clin Oncol* 2008; 38(5):347-53.
66. Park SY, Cho SH, Lee MA, et al. Diagnostic performance of MRI- versus MDCT-categorized T3cd/T4 for identifying high-risk stage II or stage III colon cancers: a pilot study. *Abdom Radiol (NY)* 2019; 44(5):1675-1685.
67. Romain C, Julien T, Jack F, et al. Microsatellite Instability in Patients With Stage III Colon Cancer Receiving Fluoropyrimidine With or Without Oxaliplatin: An ACCENT Pooled Analysis of 12 Adjuvant Trials. *Journal of Clinical Oncology* 2021; 39(6):642-651.
68. Bertagnolli MM, Niedzwiecki D, Compton CC, et al. Microsatellite instability predicts improved response to adjuvant therapy with irinotecan, fluorouracil, and leucovorin in stage III colon cancer: Cancer and Leukemia Group B Protocol 89803. *J Clin Oncol* 2009; 27(11):1814-21.
69. Klingbiel D, Saridaki Z, Roth AD, et al. Prognosis of stage II and III colon cancer treated with adjuvant 5-fluorouracil or FOLFIRI in relation to microsatellite status: results of the PETACC-3 trial. *Ann Oncol* 2015; 26(1):126-132.
70. Taieb J, Le Malicot K, Shi Q, et al. Prognostic Value of BRAF and KRAS Mutations in MSI and MSS Stage III Colon Cancer. *J Natl Cancer Inst* 2017; 109(5).
71. Andre T, Shiu KK, Kim TW, et al. Pembrolizumab in Microsatellite-Instability-High Advanced Colorectal Cancer. *N Engl J Med* 2020; 383(23):2207-2218.
72. Sinicrope FA, Ou FS, Shi Q, et al. Randomized trial of FOLFOX alone or combined with atezolizumab as adjuvant therapy for patients with stage III colon cancer and deficient DNA mismatch repair or microsatellite instability (ATOMIC, Alliance A021502). *J Clin Oncol* 2017; 35(15).
73. Grothey A, Sobrero AF, Shields AF, et al. Duration of adjuvant chemotherapy for stage III colon cancer. *New Engl J Med* 2018; 378(13):1177-1188.
74. Reinert T, Henriksen TV, Christensen E, et al. Analysis of Plasma Cell-Free DNA by Ultradeep Sequencing in Patients With Stages I to III Colorectal Cancer. *JAMA Oncol* 2019; 5(8):1124-1131.
75. Tarazona N, Gimeno-Valiente F, Gambardella V, et al. Targeted next-generation sequencing of circulating-tumor DNA for tracking minimal residual disease in localized colon cancer. *Ann Oncol* 2019; 30(11):1804-1812.
76. Tie J, Vogelstein B, Gibbs P. Circulating Tumor DNA as a Prognostic Marker in Stage III Colon Cancer-Reply. *JAMA Oncol* 2020; 6(6):932-933.
77. Guinney J, Dienstmann R, Wang X, et al. The consensus molecular subtypes of colorectal cancer. *Nat Med* 2015; 21(11):1350-6.
78. Song N, Pogue-Geile KL, Gavin PG, et al. Clinical Outcome From Oxaliplatin Treatment in Stage II/III Colon Cancer According to Intrinsic Subtypes: Secondary Analysis of NSABP C-07/NRG Oncology Randomized Clinical Trial. *JAMA Oncol* 2016; 2(9):1162-9.
79. Roepman P, Schlicker A, Tabernero J, et al. Colorectal cancer intrinsic subtypes predict chemotherapy benefit, deficient mismatch repair and epithelial-to-mesenchymal transition. *Int J Cancer* 2014; 134(3):552-62.
80. van den Berg I, van de Weerd S, Roodhart JML, et al. Improving clinical management of colon cancer through CONNECTION, a nation-wide colon cancer registry and stratification effort (CONNECTION II trial): rationale and protocol of a single arm intervention study. *BMC Cancer* 2020; 20(1):776.
81. Pages F, Mlecnik B, Marliot F, et al. International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and accuracy study. *Lancet* 2018; 391(10135):2128-2139.

82. Idos GE, Kwok J, Bonthala N, et al. The Prognostic Implications of Tumor Infiltrating Lymphocytes in Colorectal Cancer: A Systematic Review and Meta-Analysis. *Scientific Reports* 2020; 10(1):3360.
83. Sinicrope FA, Shi Q, Hermitte F, et al. Contribution of Immunoscore and Molecular Features to Survival Prediction in Stage III Colon Cancer. *JNCI Cancer Spectrum* 2020; 4(3).
84. Bernhard M, Carlo B, Gabriela B, et al. Multicenter International Society for Immunotherapy of Cancer Study of the Consensus Immunoscore for the Prediction of Survival and Response to Chemotherapy in Stage III Colon Cancer. *Journal of Clinical Oncology* 2020; 38(31):3638-3651.
85. Sinicrope FA, Graham RP. Tumor-Infiltrating Lymphocytes for Prognostic Stratification in Nonmetastatic Colon Cancer-Are We There Yet? *JAMA Oncol* 2021.
86. Pages F, Andre T, Taieb J, et al. Prognostic and predictive value of the Immunoscore in stage III colon cancer patients treated with oxaliplatin in the prospective IDEA France PRODIGE-GERCOR cohort study. *Ann Oncol* 2020; 31(7):921-929.
87. Schmoll HJ, Tabernero J, Maroun J, et al. Capecitabine plus oxaliplatin compared with fluorouracil/folinic acid as adjuvant therapy for stage III colon cancer: Final Results of the NO16968 randomized controlled phase III trial. *J Clin Oncol* 2015; 33(32):3733-3740.
88. Andre T, de Gramont A, Vernerey D, et al. Adjuvant Fluorouracil, Leucovorin, and Oxaliplatin in Stage II to III Colon Cancer: Updated 10-Year Survival and Outcomes According to BRAF Mutation and Mismatch Repair Status of the MOSAIC Study. *J Clin Oncol* 2015; 33(35):4176-87.
89. Bennouna J, André T, Campion L, et al. Rationale and Design of the IROCAS Study: Multicenter, International, Randomized Phase 3 Trial Comparing Adjuvant Modified (m) FOLFIRINOX to mFOLFOX6 in Patients With High-Risk Stage III (pT4 and/or N2) Colon Cancer—A UNICANCER GI-PRODIGE Trial. *Clin Colorectal Cancer* 2019; 18(1):e69-e73.
90. Chalabi M, Fanchi LF, Dijkstra KK, et al. Neoadjuvant immunotherapy leads to pathological responses in MMR-proficient and MMR-deficient early-stage colon cancers. *Nat Med* 2020; 26(4):566-576.
91. André T, Vernerey D, Im SA, et al. Bevacizumab as adjuvant treatment of colon cancer: updated results from the S-AVANT phase III study by the GERCOR Group. *Ann Oncol* 2020; 31(2):246-256.
92. Van KM, Greg Y, Scott K, et al. NRG-GI005 (COBRA): Phase II/III study of circulating tumor DNA as a predictive biomarker in adjuvant chemotherapy in patients with stage II colon cancer. *Journal of Clinical Oncology* 2020; 38(4_suppl):TPS261-TPS261.
93. Schraa SJ, van Rooijen KL, van der Kruijssen DEW, et al. Circulating tumor DNA guided adjuvant chemotherapy in stage II colon cancer (MEDOCC-CrEATE): study protocol for a trial within a cohort study. *BMC Cancer* 2020; 20(1):790.
94. Gayathri A, Naureen S, Clare P, et al. TRACC: Tracking mutations in cell-free DNA to predict relapse in early colorectal cancer—A randomized study of circulating tumour DNA (ctDNA) guided adjuvant chemotherapy versus standard of care chemotherapy after curative surgery in patients with high risk stage II or stage III colorectal cancer (CRC). *Journal of Clinical Oncology* 2020; 38(15_suppl):TPS4120-TPS4120.
95. Taieb J, Benhaim L, Laurent Puig P, et al. "Decision for adjuvant treatment in stage II colon cancer based on circulating tumor DNA:The CIRCULATE-PRODIGE 70 trial". *Dig Liver Dis* 2020; 52(7):730-733.

Chapter 3

Actual survival after resection of primary colorectal cancer, results from a prospective multicenter study

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ABSTRACT

Background

Colorectal cancer is the third most common type of cancer in the world. We characterize a cohort of patients who survived up to five years without recurrence and identify factors predicting the probability of cure.

Methods

We analyzed data of patients who underwent curative intent surgery for stage I-III CRC between 2007 and 2012 and who had had been included in a large multicenter study in the Netherlands. Cure was defined as 5-year survival without recurrence. Survival data were retrieved from a national registry.

Results

Analysis of data of 754 patients revealed a cure rate of 65% (n=490). Patients with stage I disease, T1- and N0-tumor had the highest probability of cure (94%, 95% and 90%, respectively). Those with a T4-tumor or N2-tumor had the lowest probability of cure (62% and 50%, respectively). A peak in the mortality rate for older patients early in follow-up suggests early excess mortality as an explanation. Patients with stage III disease, poor tumor grade, postoperative complications, sarcopenia and R1 resections show a similar trend for decrease in CSS deaths over time.

Conclusion

In the studied cohort, the probability of cure for patients with stage I-III CRC ranged from 50% to 95%. Even though most patients will be cured from CRC with standard therapy, standard therapy is insufficient for those with poor prognostic factors, such as high T- and N-stage and poor differentiation grade.

INTRODUCTION

With an incidence of over 1.8 million new cases and almost 861,000 deaths in 2018 according to the World Health Organization, colorectal cancer (CRC) is the third most common cancer in the world [1]. Currently, the American Joint Committee on Cancer (AJCC) TNM classification is the most important determinant for treatment decisions and outcome. The standard treatment for stage I-III colon cancer is surgical resection of the primary tumor for patients, which is associated with a 5-year survival rate ranging from 92% in stage 1 to 53% in stage III [2]. Still, clinical outcomes of individual patients with resectable tumors vary. Besides tumour characteristics, patient factors such as obesity, diabetes mellitus, smoking and nutritional status have been associated with survival; yet much of the disparity in prognosis remains unexplained [3-5].

Recurrence of CRC is chiefly a time-limited phenomenon, as 60–80% of recurrences becoming apparent within the first 2 years after resection, and 95% within the first 4 years after resection [6]. The chances of recurrence remote after a 5-years recurrence-free period. Although recurrence is still possible after 5 years, the medical community considers many cancers "cured" when recurrence has not occurred within 5 years after diagnosis [7]. Owing to the considerable progress in the treatment in CRC during the past few decades, more and more patients remain free from recurrent disease after surgery [8, 9]. Even though the ideal intensity of follow-up is being debated, [10, 11] the recurrence rate has been shown to reach a plateau phase 5 years after resection of the primary tumor. This is why follow-up programs in the Netherlands and many other countries have been limited to 5 years [10, 12-14]. In this multi-center study in a large Dutch colorectal cancer population, we sought to characterize the patients who survive up to five years without recurrence of disease and identify factors that affect probability of cure.

METHODS

Study population

We analyzed data of patients with stage I-III colorectal cancer who had undergone curative intent surgery and had between 2007 and 2012 been enrolled in the MATCH-study, a prospective observational cohort study in patients undergoing curative resection for primary colorectal cancer in seven centers in the region of Rotterdam, the Netherlands [15]. The purpose of the MATCH study was to identify subtypes of colorectal cancer, related prognostic markers and outcome of treatment [16]. The MATCH study was approved by the Erasmus MC medical ethics review board (MEC-2007-088) and all patients provided written informed consent. All patients enrolled between 2007 and 2012 had the potential for 5 years of follow-up.

Patient work-up and follow-up

Work-up

All patients underwent colonoscopy with a biopsy of any suspicious lesions. After tissue diagnosis was confirmed, laboratory studies were done with a goal of assessing patients' organ function (liver, kidneys) in anticipation of diagnostic and therapeutic procedures and also to estimate tumor burden. Adequate imaging of the chest and abdomen was obtained for staging purposes. For colon cancer patients this consisted of CT-abdomen and X-thorax and ultrasound of the liver when indicated. For rectal cancer patients this consists of CT-thorax/abdomen and MRI rectum/pelvis.

Further workup was driven by clinical setting, patient functional status and comorbidities and presenting symptoms. After adequate staging, adjuvant chemotherapy was offered for patients with high risk stage II and stage III colon cancer. At the time of this study, standard treatment constisted of 6 months CAPOX or FOLFOX. For rectal cancer patients, preoperative radiotherapy was offered for patients with T2-T4 tumors. For rectal cancer patients with positive CRM, or ≥4 positive lymphnodes, a combination of neoadjuvant radiotherapy and chemotherapy was offered.

Follow-up

CEA monitoring was performed 3-to-6 monthly in the first 3 years and 6-to-12 monthly hereafter. Ultrasound of the liver or an abdominal CT every 6 months in the first 1-2 years and yearly hereafter. For rectal cancer patients, an additional x-thorax or CT-thorax could be considered, depending on stage.

Observed cure and follow-up status

Observed cure was defined as actual 5-year survival with no recurrence [6, 7, 13, 14]. At last follow up, a patient was classified as having no evidence of disease (NED) if having survived without documented recurrence, and as having died of disease (DOD) if the cause of death was listed as cancer in the national death registry. A patient classified as death of other cause (DOC) if a clearly attributable non-cancer reason for death was mentioned in the registry of Statistics Netherlands (*Centraal Bureau voor de Statistiek;* CBS). A patient was classified as dead of unknown cause (DUC) if no identifiable cause of death was found in the registry of Statistics Netherlands. A five-year survivor with evidence of recurrent disease in the medical record was classified as alive with disease (AWD) [17].

Predictors and outcome measures

Demographic variables included age and gender. Clinical variables included BMI, American Society of Anesthesiologists (ASA) score, International Union Against Cancer tumour node metastasis (TNM) classification of malignant tumours, tumor differentiation grade, tumor location, comorbidities, charlson comorbidity index, sarcopenia [18] . Treatment variables included: radicality, (neo)adjuvant therapy, postoperative complications classified according to Clavien-Dindo and readmissions <30 days.

To identify characteristics that may preclude long-term survival and cure, we compared the frequencies of these factors between specific survival cohorts defined as less than 1, 1 to 3, 3 to 5 and more than 5 years [17].

Cancer specific survival (CSS) was calculated from the day of surgery to the day of death (from disease) or loss to follow-up, whichever came first. Date and cause of death were obtained from the national registry of Statistics Netherlands (*Centraal Bureau voor de Statistiek;* CBS). Patients who died of other causes than CRC were censored at the date of last follow-up. CSS was estimated using Kaplan-Meier methods and compared using log-rank.

Statistical methods

The standard Cox proportional hazard model assumes proportional hazards, an assumption that can fail when survival curves have plateaus at the tails [19]. Hence, a semi-parametric proportional hazards mixture cure model was used to estimate the probability of cure and assess differences in outcome between cured patients and those who were not cured. In this model, the probability of being cured was modelled with logistic regression and the survival probability for patients who experienced the event of interest was estimated using a proportional hazards model [20-22]. All analyses were performed using the smcure package in R. v.3.3.2 (R foundation for Statistical Computing, Vienna, Austria) [22]. Two-sided *p*-values <0.05 were considered statistically significant.

RESULTS

Patient characteristics and follow-up status

A total of 754 patients included in the MATCH study underwent surgical resection with curative intent in the period 2007 through 2012 (Figure 1). At last follow-up, 117 patients (15.2%) could be classified as DOD, 40 (5.3%) as AWD, and 11 (1.5%) as AUD. In total, 93 patients (12.3%)

could be classified as DOC and 29 (3.8%) as DUC. Data of the latter were excluded from CSS analyses. After 5 years of follow-up, 464 patients (61.5%) could be classified as NED and 26 (3.4%) died of a non-cancer related cause (DOC). These patients are considered cured from disease (NED+DOC>5Y; n=490). (Figure 1).

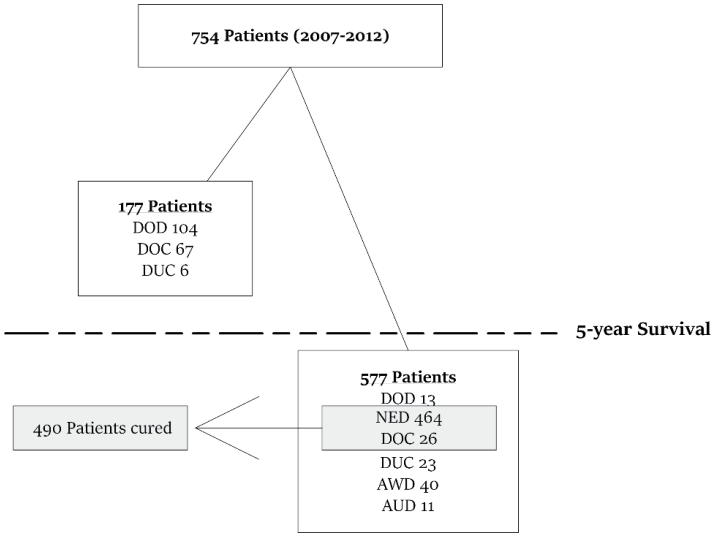


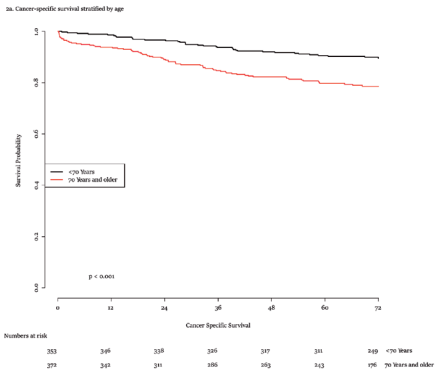
Figure 1. Current status and observed cure in study population

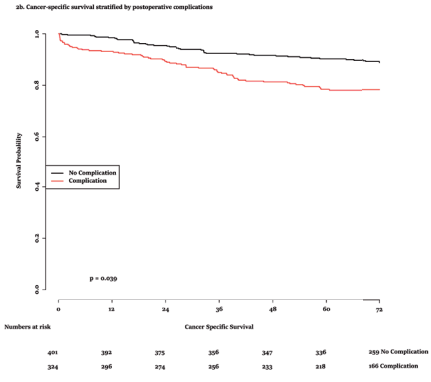
Cancer specific survival through follow-up

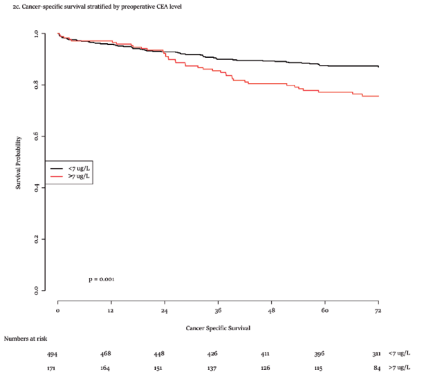
Table 1 reports descriptive analyses on characteristics of patients over time. Patients were grouped by survival into less than 1 year survival, 1–3 years, 3–5 years, more than 5-years survival, and an additional category cured. The first three groups include only DOD patients; the non-cured group >5 years consists of AWD, DOD and AUC patients. The cured patients included 52.2% men, and 42.9% were aged ≥70. The latter relatively more often had died of CRC in the early years following surgery than had patients <70 years, as can be seen in table 1 from the decreasing proportion of older aged patients dying of CRC over time and increasing proportions of younger patients dying over time. Patients with stage III colon cancer, poor tumor grade, postoperative complications, sarcopenia and or an incomplete resection margin show a similar trend for decrease in CSS deaths over time (Table 1).

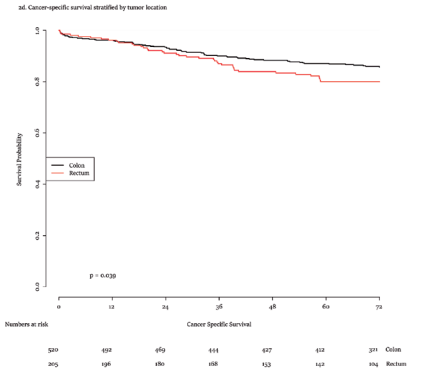
Kaplan Meier survival analyses were performed to determine which characteristics were associated with CSS. It appeared that age >70, ($p<0.001$), preoperative CEA level ≥7ug/L ($p=0.001$), high T-stage ($p<0.001$), high N-stage ($p<0.001$), high tumor stage ($p<0.001$), poor tumor differentiation ($p=0.010$), rectal cancer ($p=0.039$) and occurrence of postoperative complications

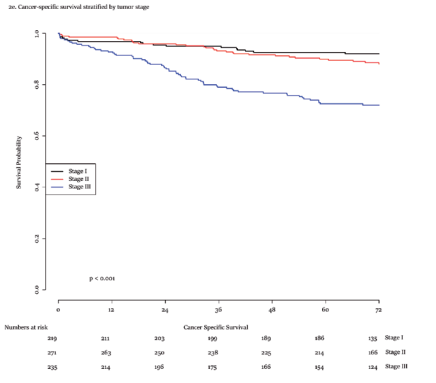
(p=0.039) were all significantly associated with shorter CSS (Table 1; Figure 2). It should be noted that from the patients with an N0 tumor, a total of 85 patients had <10 lymphnodes dissected which could have led to wrong nodal staging.

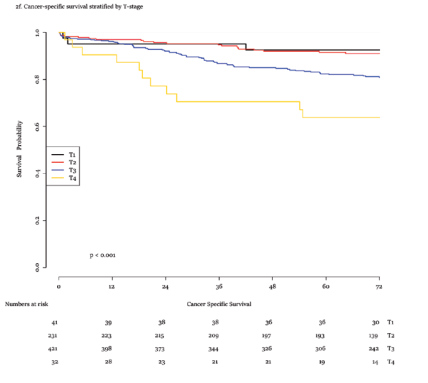












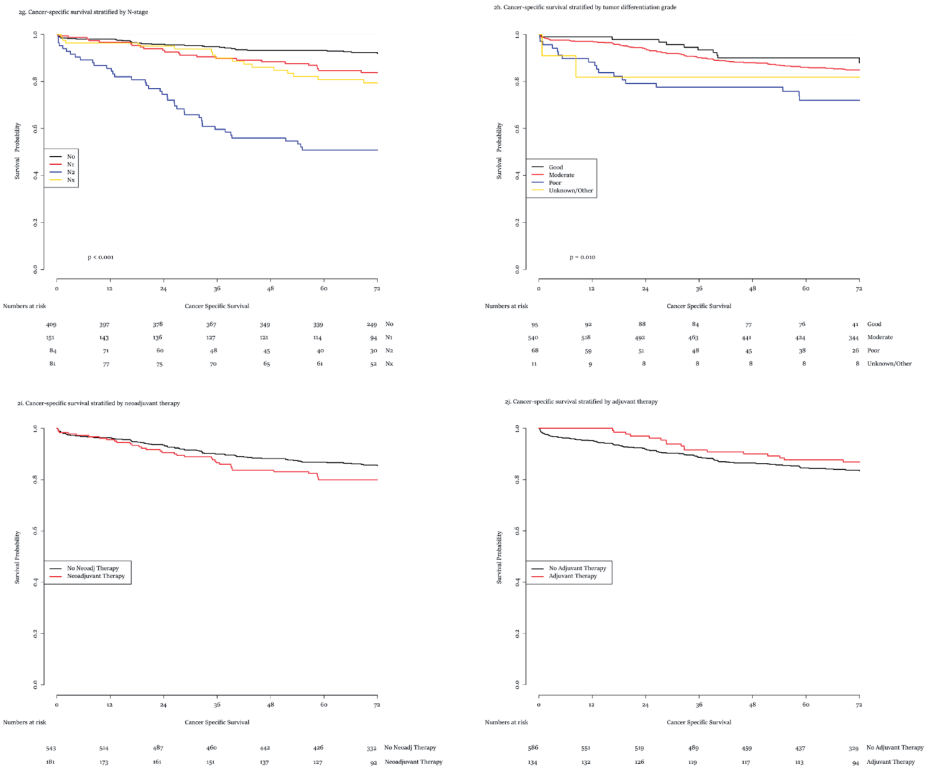


Figure 2. Kaplan-Meier curves on cancer-specific survival stratified by prognostic clinical factors. **a** Cancer-specific survival stratified by age. **b** Cancer-specific survival stratified by postoperative complications. **c** Cancer-specific survival stratified by preoperative CEA level. **d** Cancer-specific survival stratified by tumor location. **e** Cancer-specific survival stratified by tumor stage. **f** Cancer-specific survival stratified by T-stage. **g** Cancer-specific survival stratified by N-stage. **h** Cancer-specific survival stratified by tumor differentiation grade. **i** Cancer-specific survival stratified by neoadjuvant therapy. **j** Cancer-specific survival stratified by adjuvant therapy

Table 1. Comparison of Prognostic Factors Among Survival Cohorts

	0-1	%	1-3	%	3-5	%	>5*	%	Cured	%	Missing %
Gender											0
Male	18	(64.3)	31	(63.3)	13	(48.1)	34	(53.1)	256	(52.2)	
Female	10	(35.7)	18	(36.7)	14	(51.9)	30	(46.9)	234	(47.8)	
Age											0
<70	5	(17.9)	17	(34.7)	11	(40.7)	31	(48.4)	280	(57.1)	
≥70	23	(82.1)	32	(65.3)	16	(59.3)	33	(51.6)	210	(42.9	
BMI											1.1
1 (<18.5)	1	(3.7)	1	(2.0)	1	(3.7)	0	(0.0)	15	(3.1)	
2 (18.5 – 24.9)	10	(37.0)	22	(44.9)	10	(37.)	20	(32.3)	181	(37.3)	
3 (≥25.0)	16	(59.3)	26	(53.1)	16	(59.3)	42	(67.7)	289	(59.6)	

Table 1. Comparison of Prognostic Factors Among Survival Cohorts (continued)

	0-1	%	1-3	%	3-5	%	>5*	%	Cured	%	Missing %
Sarcopenia											18.8
No	7	(31.8)	15	(41.7)	10	(47.6)	23	(50.0)	211	(51.8)	
Yes	15	(68.2)	21	(58.3)	11	(52.4)	23	(50.0)	196	(48.2)	
Low muscle density											19.6
No	5	(23.8)	11	(40.6)	6	(28.6)	13	(29.5)	159	(39.4)	
Yes	17	(76.2)	25	(69.4)	15	(71.4)	31	(70.5)	245	(60.6)	
Sarcopenia + obese											0
No	26	(92.9)	44	(89.8)	26	(96.3)	63	(98.4)	465	(94.9)	
Yes	2	(7.1)	5	(10.2)	1	(3.7)	1	(1.6)	25	(5.1)	
Diabetes Mellitus											0.1
No	22	(78.6)	41	(83.7)	20	(76.9)	48	(75.0)	402	(82.0)	
Yes	6	(21.4)	8	(16.3)	6	(23.1)	16	(25.0)	88	(18.0)	
Congestive heart failure											0
No	25	(89.3)	45	(91.8)	24	(88.9)	63	(98.3)	466	(95.1)	
Yes	3	(10.7)	4	(8.2)	3	(11.1)	1	(1.6)	24	(4.9)	
COPD											0
No	27	(96.4)	44	(89.8)	23	(85.2)	62	(96.9)	454	(92.7)	
Yes	1	(3.6)	5	(10.2)	4	(14.8)	2	(3.1)	26	(7.3)	
Charlson comorbidity index											0.4
0	11	(39.3)	23	(46.9)	10	(38.5)	39	(60.9)	266	(54.4)	
1+	17	(60.7)	26	(53.1)	16	(61.5)	25	(39.1)	223	(45.6)	
ASA-score											
I - II	22	(78.6)	45	(91.8)	20	(74.1)	54	(85.7)	409	(84.2)	
III - IV	6	(21.4)	4	(8.2)	7	(25.9)	9	(14.3)	77	(15.8)	
CEA											8.6
<7ug/L	21	(90.8)	28	(59.6)	11	(45.8)	36	(62.1)	360	(79.5)	
≥7ug/L	5	(19.2)	19	(40.4)	13	(54.2)	22	(37.9)	93	(20.5)	
Tumor location											
Colon	20	(71.4)	31	(63.3)	14	(51.9)	41	(64.1)	371	(75.7)	0
Rectum	8	(28.6)	18	(36.7)	13	(48.1)	23	(35.9)	119	(24.3)	
T-stage											0
1	2	(7.1)	0		1	(3.7)	2	(3.1)	34	(6.9)	
2	7	(25)	5	(10.2)	7	(25.9)	22	(34.3)	171	(34.9)	
3	16	(57.1)	38	(77.6)	17	(63)	36	(56.2)	270	(55.1)	
4	3	(10.7)	6	(12.2)	2	(7.4)	4	(6.2)	15	(3.1)	

Table 1. Comparison of Prognostic Factors Among Survival Cohorts (continued)

	0-1	%	1-3	%	3-5	%	>5*	%	Cured	%	Missing %
N-stage											0
0	8	(28.6)	13	(26.5)	6	(22.2)	36	(56.2)	303	(61.8)	
1	5	(17.9)	10	(20.4)	7	(25.9)	11	(17.2)	103	(21)	
2	12	(42.9)	21	(42.9)	7	(25.9)	7	(25.9)	33	(6.7)	
X	**3**	(10.7)	5	(10.4)	7	(25.9)	10	(15.6)	51	(10.4)	
Tumor stage											0
I	7	(25.0)	4	(8.2)	5	(18.5)	22	(34.4)	164	(33.5)	
II	4	(14.3)	14	(28.6)	8	(29.6)	24	(37.5)	190	(38.8)	
III	17	(60.7)	31	(63.3)	14	(51.9)	18	(28.1)	136	(27.8)	
Tumor grade											1.5
Good	1	(3.7)	5	(10.4)	3	(11.1)	10	(15.9)	66	(13.7)	
Moderate	16	(59.3)	36	(75.0)	21	(77.8)	47	(74.6)	377	(78.1)	
Poor	8	(29.6)	7	(14.6)	3	(11.1)	5	(7.9)	33	(6.8)	
Unknown/Other	2	(7.4)	0		0		1	(1.6)	7	(1.4)	
Radicality											0.3
R0	26	(92.6)	48	(98.0)	27	(100.0)	63	(98.4)	477	(97.5)	
R1	2	(7.1)	1	(2.0)	0		1	(1.6)	12	(2.5)	
Postoperative complications											0
No	6	(21.4)	24	(49.0)	8	(29.6)	38	(59.4)	298	(60.8)	
Yes	22	(78.6)	25	(51.)	19	(70.4)	26	(40.6)	193	(39.2)	
Readmission <30 days											0.1
No	24	(88.9)	47	(95.9)	23	(85.2)	55	(85.9)	438	(89.4)	
Yes	3	(11.1)	2	(4.1)	4	(14.8)	9	(14.1)	52	(10.6)	
Neoadjuvant chemotherapy											0
No	20	(71.4)	33	(59.3)	16	(59.3)	43	(67.2)	383	(78.3)	
Yes	8	(28.6)	16	(32.7)	11	(40.7)	21	(32.8)	106	(21.7)	
Adjuvant chemotherapy											0
No	28	(100)	37	(77.1)	22	(81.5)	55	(85.9)	382	(78.6)	
Yes	0		11	(22.9)	5	(18.5)	9	(14.1)	104	(21.4)	

* non-cured group >5 years consists of AWD, DOD and AUC patients

Observed cure and predicted cure

The potential survival cohort in Table 2 includes the 621 patients categorized as NED, DOD or AWD. Overall, 577 patients survived 5 years. Most of them had been classified as NED (n = 464, 80.4%), and a small minority (n=26, 4.5%) as DOC. These two groups are considered cured from disease; thus, the observed cure rate was 65% (490/754). At 5 years follow up,

40 patients were classified as AWD and 13 as DOD. Data on recurrence were missing for 11 patients alive at 5 years (AUD). The CSS for the whole study cohort is visualized by a Kaplan-Meier curve (Figure 3).

The observed cure rate in patients aged ≥70 years was 74.7%, versus 82.3% for patients aged <70 years. The observed cure rate for women was slightly higher than that for men (81.8% versus 76.4%). The observed cure rate for patients with a T4-tumor was notably low at 50%, and considerably higher for patients with a T1-tumor (94.4%), T2-tumor (88.6%) and even those with a T3-tumor (75.6%). Patients with a N2-tumor had the lowest observed cure rate, viz. only 41.3%, as opposed to 90.2% for patients with N0-tumor and 76.9% for patients with an N1-tumor. The observed cure rate for patients with stage III CRC was 63.6%, almost 30% lower than that for patients with stage I disease (91.1%). Furthermore, the observed cure rate for patients with a poor tumor grade was only 62% – in line with that for patients with CEA ≥7ug/L (64%) and patients with rectal cancer (67%).

The predicted cure rate of 80.1% for patients ≥ 70 years of age is slightly higher than the observed cure rate for this group (Table 2). Patients with a T1- and/or N0-tumor had the highest probability of cure, i.e., 94.4% and 90.2%, respectively. Conversely, patients with a

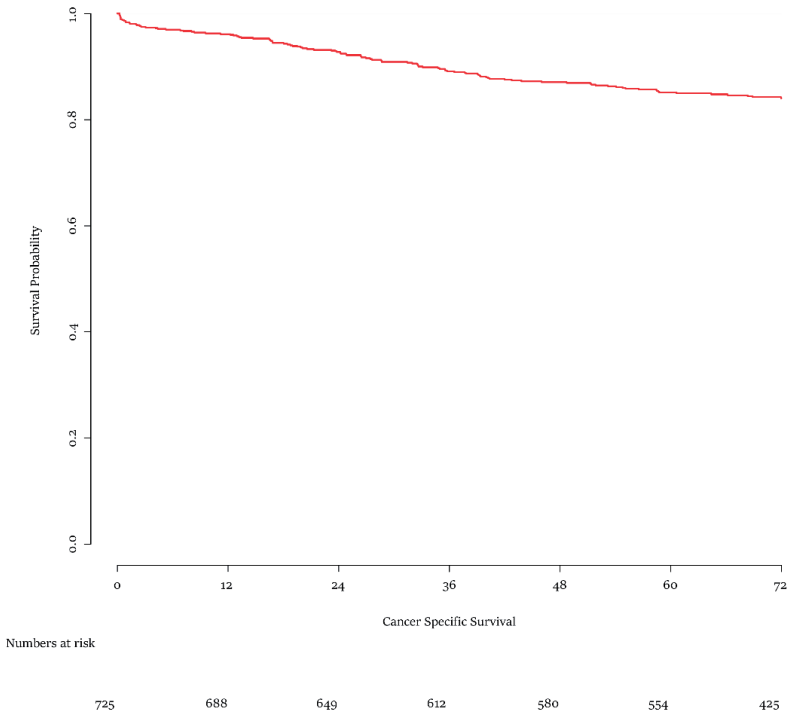


Figure 3. Kaplan-Meier curve for cancer-specific survival for all patients undergoing resection for stage I–III colorectal cancer

T4-tumor or N2-tumor had the lowest probability of cure; i.e., 62.3% and 50.1%, respectively. The predicted probability of cure for tumor stage was 94% for stage I, 88% for stage II, and 71% for stage III. Regarding type of cancer, colon cancer was associated with a higher probability of cure than is rectal cancer (85.5% versus 80%). Two other factors were associated with a relatively low probability of cure; i.e., preoperative CEA level of >7uq/L and poor tumor differentiation (both 73%). The predicted cure rate of patients who experienced postoperative complications was 76.8%. Although underweight BMI and diabetes were not significantly

Table 2. Characteristics of patients with potential cure and probability of cure estimated from the semiparametric mixture cure model

	Total NED, DOD, AWD (N=621)	Observed 5-y survivors	% Observed	Predicted Cure
Gender				
Male	335	256	76.4	82
Female	286	234	81.8	83.7
Age				
<70	340	280	82.4	88.5
≥70	281	210	74.7	80.1
BMI				
1 (< 18.5)	17	15	88.2	75.6
2 (18.5 - 24.9)	228	181	79.4	85.5
3 (≥ 25.0)	368	289	87.5	84
Sarcopenia				
No	254	211	83.1	86
Yes	246	196	79.7	82.8
Low muscle density				
No	190	159	83.7	86.7
Yes	305	245	80.3	83
Sarcopenia + obesity				
No	590	465	85	75.4
Yes	31	25	75.4	85.7
Diabetes Mellitus				
No	508	402	79.5	86.6
Yes	114	88	77.2	75.8
Decompensatio Cordis				
No	591	466	78.9	84.8
Yes	30	24	80	78.7
COPD				
No	591	454	78.41	84.9
Yes	42	36	80	78.2

Table 2. Characteristics of patients with potential cure and probability of cure estimated from the semiparametric mixture cure model (continued)

	Total NED, DOD, AWD (N=621)	Observed 5-y survivors	% Observed	Predicted Cure
Charlson comorbidity index				
0	336	266	79.2	88.8
1+	283	223	78.9	78.8
ASA-score				
I+II	526	409	78.1	83.7
III+IV	92	77	83.7	87.5
CEA				
<7ug/L	430	360	83.7	87.8
≥7ug/L	146	93	63.7	73.1
Tumor location				
Colon	444	371	83.6	85.8
Rectum	177	119	67.2	80
T-stage				
1	36	34	94.4	92.2
2	193	171	88.6	92.1
3	362	270	74.6	81.2
4	30	15	50	62.3
N-stage				
0	336	303	90.2	92.7
1	134	103	76.9	82.9
2	80	33	41.3	50.1
X	71	51	71.8	83.4
Tumor stage				
I	180	164	91.1	94.6
II	227	190	83.7	88.4
III	214	136	63.6	71
Tumor grade				
Good	79	66	83.5	86.5
Moderate	470	377	80.2	85.7
Poor	53	33	62.3	73.1
Unknown/Other	10	7	70	79.7
Radicality				
R0	605	477	80	84.5
R1	15	12	78.8	77.5

Table 2. Characteristics of patients with potential cure and probability of cure estimated from the semiparametric mixture cure model (continued)

	Total NED, DOD, AWD (N=621)	Observed 5-y survivors	% Observed	Predicted Cure
Postoperative complications				
No	266	298	83.9	90.4
Yes	355	192	72.2	76.8
Readmission <30 days				
No	552	438	79.5	94.1
Yes	68	52	76.5	87.1
Neoadjuvant therapy				
No	462	383	82.9	85.7
Yes	158	106	67.1	79.9
Adjuvant chemotherapy				
No	127	382	78.1	84
Yes	489	104	81.9	86.1

associated with CSS in univariate analyses, both had a relatively low probability of cure in the mixture cure model (75.6% and 73.1%, respectively). Nonetheless, patients who did not have postoperative complications and had not been readmitted within <30 days postoperatively, had a probability of cure of over 90% (90.4% and 94.1%, respectively).

DISCUSSION

The findings of this multicenter cohort study are consistent with failure of the curative intent treatment strategies for stage I-III CRC in 35% of cases. Age >70 years at diagnosis, high preoperative CEA level, rectal cancer, high T-and N-stage, high tumor stage, poor tumor differentiation and postoperative complications were all individual poor prognostic factors for cancer-specific survival after surgery for stage I-III CRC. Observations and mixture cure model analysis showed that patients with T4-stage, N2-stage, stage III CRC, CEA level ≥7ug/L and poor tumor differentiation had the lowest chance of eventual cure. Nevertheless, the clear majority of 5-year survivors (65%) had no evidence of disease or had died of a non-cancer related cause, and could therefore be defined as cured Patients with a T1-stage tumor, N0-stage tumor, tumor stage I and/or postoperative complications had the highest probability of cure (>90%).

In the Netherlands and most other countries, once a patient has remained free from recurrence of disease for 5 years after surgery, the medical community considers many cancers “cured” [7].

Although recurrence of disease after 5 years is not impossible, the probability of this happening is very low. Therefore, follow-up programs are usually limited to 5 year postoperatively [10, 12].

We found that the pathologic tumor characteristics were the most important indicators of probability of cure. The probability of cure for patients with a T1-tumor was 92%, which decreased to 62% for patients with a T4-tumor. Correspondingly, the probability of cure for patients with an N0-tumor was 93%, which decreased markedly to 50% for patients with an N2-tumor. These findings are in line with previous research by Gunderson and colleagues, who showed a 5-year survival rate of 97% for both patients with a T1N0 tumor and patients with T2N0 tumor, compared with 55% for patients with a T4N0 tumor and 56.8% for patients with a T1N2 tumor [23]. Tumor grade is generally considered a stage-independent prognostic factor for survival, in that poor differentiated tumors are associated with poor patient survival [24, 25]. In the current study, poorly differentiated tumors were associated with a significantly lower survival rate (62%) and predicted cure (73%) than were well-differentiated tumors (83% and 87% respectively). These results highlight the importance of especially T- and N-staging in non-metastatic CRC, seeing that current treatment strategies that have a curative intent are insufficient for a subgroup of patients with poor tumor characteristics. Some studies have found encouraging survival outcomes in patients with a T4-tumor with the use of proactive strategies, such as the second-look approach [26, 27] and prophylactic resection of target organs for peritoneal metastases during the first surgery [28]. However, two large phase III trials failed to show benefit from adjuvant intraperitoneal hyperthermic chemoperfusion (HIPEC) in high-risk patients [29, 30]. An effective treatment for high-risk patients is therefore still needed.

The present study findings complement earlier results in in that they associate older age with poorer cancer-specific survival [31, 32]. Provision of less intensive therapy to the elderly or the elderly refusing treatment may have resulted in higher recurrence rates and causal death [33-36].

In line with previous literature, patients with rectal cancer had significantly lower chances of long-term survival and cure than had patients with colon cancer [37]. Sex, for which literature shows contradicting results, was not a prognostic factor for survival [38-42]. Furthermore, we found no association between the presence of comorbidities and CSS. Diabetes, congestive heart failure and COPD, but also grading-systems for comorbidities such as the ASA-score and the CCI-index were not associated with CSS in our study. The literature on the association between comorbidities and CSS is somewhat contradictory. While some studies found a lower survival with increasing comorbidity, other studies found the association differs between colon cancer and rectal cancer [43, 44]. The reasons underlying these results in other studies have not been elucidated, although possible contributors include under-treatment and reduced resilience to cope with cancer effects and treatment toxicity.

This is, to our knowledge, the first study to provide unique estimates of the likelihood of both observed and predicted cure depending on particular risk factors in a large Dutch prospective multicenter study on stage I-III colorectal cancer patients.

However, this study has several limitations. In general, surveillance imaging had been performed at least every 6 months after surgery. The time interval of 6 months may have led to lead time bias. As mentioned in the results, for 85 patients with an N0 tumor, less than 10 lymphnodes were dissected or pathologically analyzed which could have led to wrong nodal staging. This could inherently lead to survival differences if some of these patients did actually have lymphnode metastases. Furthermore, we did not address molecular tumor characteristics that are related to survival, which play an increasingly bigger role in the prediction of survival in CRC [45].

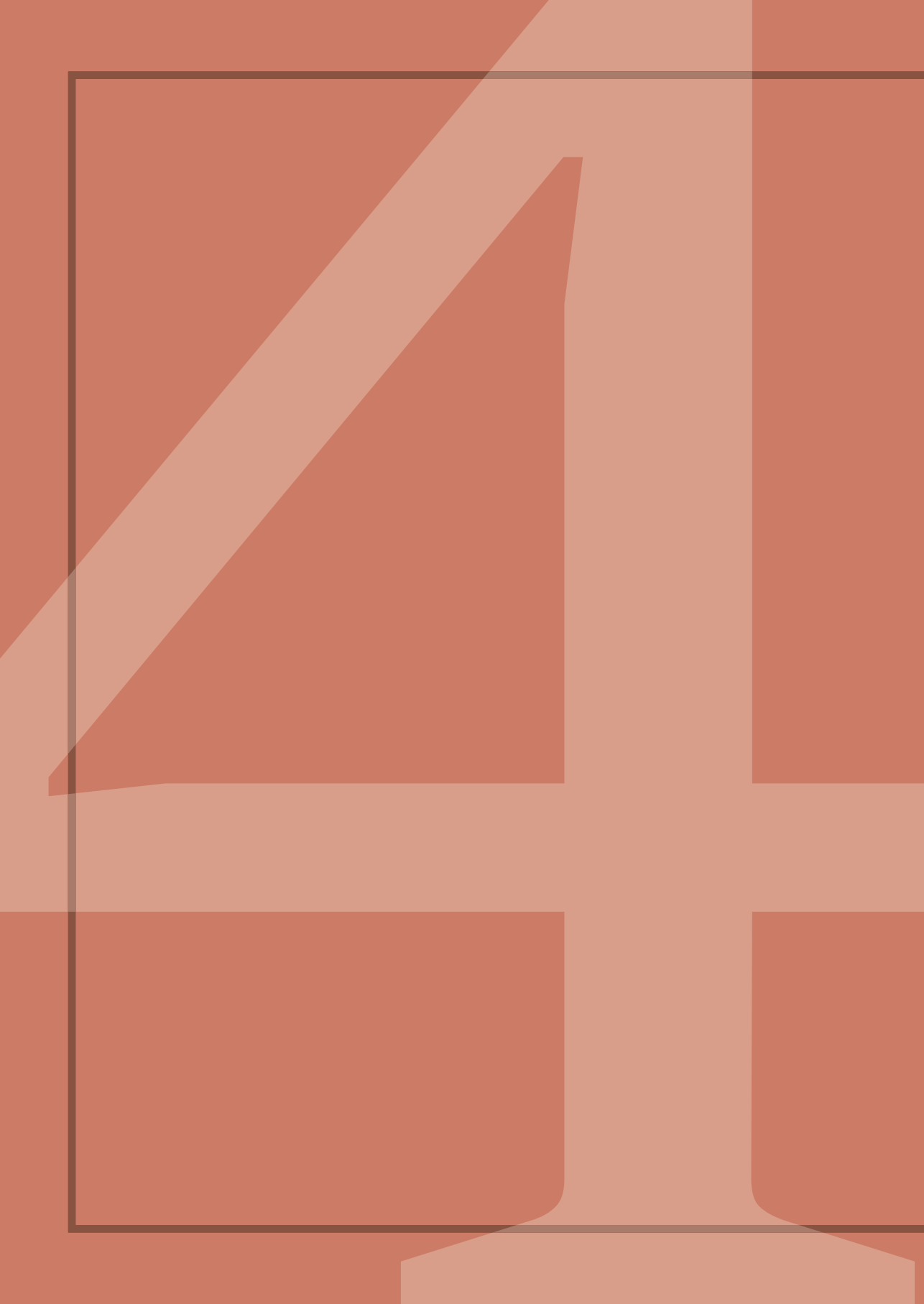
In conclusion, while CRC is recognized as a possible fatal malignancy, a substantial improvement on therapies and thereby survival of patients with CRC has been accomplished over the recent years. Appropriate survival analysis like the mixture cure rate model performed in this study can help the clinicians and researchers in identifying potential risk factors, which affect the survival and cure fraction of patients who are not susceptible to death from CRC. This mixture cure model provides a framework to compare both patient-related and treatment-related prognostic factors and to gives valuable insight in the probability of being cured of CRC for each of these variables. The probability of cure for patients with stage I-III colorectal cancer included in this study ranges from 50% to 94%. Even with poor prognostic factors, such as high tumor stage and poor differentiation grade, cure is highly likely with standard therapy consisting of surgery and adjuvant or neoadjuvant systemic therapy when indicated. Still, this is less obvious for older patients with high T- and N-stage tumors and/or poor tumor differentiation. Instead of only providing patients with overall 5-year survival rates, with general patient characteristics, this cure model can aid physicians in providing a more individualized prognosis and chance of curation from this disease.

REFERENCES

1. organisation Wh. The Global Cancer Observatory (GCO) 2018. Available at: https://gco.iarc.fr/.
2. Dienstmann R, Mason MJ, Sinicrope FA, et al. Prediction of overall survival in stage II and III colon cancer beyond TNM system: a retrospective, pooled biomarker study. *Ann Oncol* 2017; 28(5):1023-1031.
3. Karahalios A, English DR, Simpson JA. Weight change and risk of colorectal cancer: a systematic review and meta-analysis. *Am J Epidemiol* 2015; 181(11):832-45.
4. Lauby-Secretan B, Scoccianti C, Loomis D, et al. Body Fatness and Cancer--Viewpoint of the IARC Working Group. *N Engl J Med* 2016; 375(8):794-8.
5. Yuhara H, Steinmaus C, Cohen SE, et al. Is diabetes mellitus an independent risk factor for colon cancer and rectal cancer? *Am J Gastroenterol* 2011; 106(11):1911-21; quiz 1922.
6. Longo WE, Johnson FE. The preoperative assessment and postoperative surveillance of patients with colon and rectal cancer. *Surg Clin North Am* 2002; 82(5):1091-108.
7. Society AC. Cancer Facts & Figures 2020. *American Cancer Society journal, CA: A Cancer Journal for Clinicians* 2020
8. Rondeau V, Schaffner E, Corbiere F, et al. Cure frailty models for survival data: application to recurrences for breast cancer and to hospital readmissions for colorectal cancer. *Stat Methods Med Res* 2013; 22(3):243-60.
9. Lambert PC, Thompson JR, Weston CL, et al. Estimating and modeling the cure fraction in population-based cancer survival analysis. *Biostatistics* 2007; 8(3):576-94.
10. Primrose JN, Perera R, Gray A, et al. Effect of 3 to 5 years of scheduled CEA and CT follow-up to detect recurrence of colorectal cancer: the FACS randomized clinical trial. *JAMA* 2014; 311(3):263-70.
11. Wille-Jørgensen P, Syk I, Smedh K, et al. Effect of More vs Less Frequent Follow-up Testing on Overall and Colorectal Cancer–Specific Mortality in Patients With Stage II or III Colorectal Cancer: The COLOFOL Randomized Clinical Trial. *JAMA* 2018; 319(20):2095-2103.
12. Dutch Cancer Institute. Colorectal cancer guideline. 2017.
13. Yu Y, Carey M, Pollett W, et al. The long-term survival characteristics of a cohort of colorectal cancer patients and baseline variables associated with survival outcomes with or without time-varying effects. *BMC Medicine* 2019; 17(1):150.
14. Bouvier A-M, Launoy G, Bouvier V, et al. Incidence and patterns of late recurrences in colon cancer patients. *International Journal of Cancer* 2015; 137(9):2133-2138.
15. Lalmahomed ZS, Coebergh van den Braak RRJ, Oomen MHA, et al. Multicenter fresh frozen tissue sampling in colorectal cancer: does the quality meet the standards for state of the art biomarker research? *Cell Tissue Bank* 2017; 18(3):425-431.
16. Kloosterman WP, Coebergh van den Braak RRJ, Pieterse M, et al. A systematic analysis of oncogenic gene fusions in primary colon cancer. *Cancer Res* 2017.
17. Creasy JM, Sadot E, Koerkamp BG, et al. Actual 10-year survival after hepatic resection of colorectal liver metastases: what factors preclude cure? *Surgery* 2018; 163(6):1238-1244.
18. van Vugt JLA, Buettner S, Levolger S, et al. Low skeletal muscle mass is associated with increased hospital expenditure in patients undergoing cancer surgery of the alimentary tract. *PLoS One* 2017; 12(10):e0186547.
19. Othus M, Barlogie B, Leblanc ML, et al. Cure models as a useful statistical tool for analyzing survival. *Clin Cancer Res* 2012; 18(14):3731-6.

20. Yilmaz YE, Lawless JF, Andrulis IL, et al. Insights from mixture cure modeling of molecular markers for prognosis in breast cancer. *J Clin Oncol* 2013; 31(16):2047-54.
21. Sy JP, Taylor JM. Estimation in a Cox proportional hazards cure model. *Biometrics* 2000; 56(1):227-36.
22. Fit Semiparametric Mixture Cure Models [computer program]. 2012.
23. Gunderson LL, Jessup JM, Sargent DJ, et al. Revised TN categorization for colon cancer based on national survival outcomes data. *J Clin Oncol* 2010; 28(2):264-71.
24. Blenkinsopp WK, Stewart-Brown S, Blesovsky L, et al. Histopathology reporting in large bowel cancer. *J Clin Pathol* 1981; 34(5):509-13.
25. Schneider NI, Langner C. Prognostic stratification of colorectal cancer patients: current perspectives. *Cancer Manag Res* 2014; 6:291-300.
26. Serrano Del Moral A, Perez Viejo E, Manzanedo Romero I, et al. Systematic Second-look Surgery Plus HIPEC in Patients Without Evidence of Recurrence, at High Risk of Carcinomatosis After Colorectal Cancer Resection. *Cir Esp* 2018; 96(2):96-101.
27. Elias D, Honoré C, Dumont F, et al. Results of systematic second-look surgery plus hipec in asymptomatic patients presenting a high risk of developing colorectal peritoneal carcinomatosis. *Ann Surg* 2011; 254(2):289-293.
28. Sammartino P, Sibio S, Biacchi D, et al. Long-term results after proactive management for locoregional control in patients with colonic cancer at high risk of peritoneal metastases. *Int J Colorectal Dis* 2014; 29(9):1081-9.
29. Moran BJ. PROPHYLOCHIP: no benefit of second-look surgery plus HIPEC for colorectal peritoneal metastases. *Lancet Oncol* 2020; 21(9):1124-1125.
30. Charlotte K DD, Cornelis J, Petur S, Johannes C, Arend A, Bemelman WA, Brandt A, Bremers A, Burger J, van Grevernstein W, Hemmer P, de Hingh I, Kok N, Tuynman J, Wiezer M, de Wilt J, Dijkgraaf M, Tanis P. Adjuvant HIPEC in patients with colon cancer at high risk of peritoneal metastases: Primary outcome of the COLOPEC multicenter randomized trial. . *J Clin Oncol* 2019; 37:482–482:482.
31. Baghestani AR, Daneshva T, Pourhoseingholi MA, et al. Survival of Colorectal Cancer in the Presence of Competing- Risks - Modeling by Weibull Distribution. *Asian Pac J Cancer Prev* 2016; 17(3):1193-6.
32. Morrison DS, Parr CL, Lam TH, et al. Behavioural and metabolic risk factors for mortality from colon and rectum cancer: analysis of data from the Asia-Pacific Cohort Studies Collaboration. *Asian Pac J Cancer Prev* 2013; 14(2):1083-7.
33. Cree M, Tonita J, Turner D, et al. Comparison of treatment received versus long-standing guidelines for stage III colon and stage II/III rectal cancer patients diagnosed in Alberta, Saskatchewan, and Manitoba in 2004. *Clin Colorectal Cancer* 2009; 8(3):141-5.
34. Winget M, Hossain S, Yasui Y, et al. Characteristics of patients with stage III colon adenocarcinoma who fail to receive guideline-recommended treatment. *Cancer* 2010; 116(20):4849-56.
35. Lemmens VE, van Halteren AH, Janssen-Heijnen ML, et al. Adjuvant treatment for elderly patients with stage III colon cancer in the southern Netherlands is affected by socioeconomic status, gender, and comorbidity. *Ann Oncol* 2005; 16(5):767-72.
36. Quipourt V, Jooste V, Cottet V, et al. Comorbidities alone do not explain the undertreatment of colorectal cancer in older adults: a French population-based study. *J Am Geriatr Soc* 2011; 59(4):694-8.

37. van Erning FN, van Steenbergen LN, Lemmens V, et al. Conditional survival for long-term colorectal cancer survivors in the Netherlands: who do best? *Eur J Cancer* 2014; 50(10):1731-1739.
38. Azizmohammad Looha M, Pourhoseingholi MA, Nasserinejad M, et al. Application of a non-parametric non-mixture cure rate model for analyzing the survival of patients with colorectal cancer in Iran. *Epidemiol Health* 2018; 40:e2018045.
39. Paulson EC, Wirtalla C, Armstrong K, et al. Gender influences treatment and survival in colorectal cancer surgery. *Dis Colon Rectum* 2009; 52(12):1982-91.
40. McArdle CS, McMillan DC, Hole DJ. Male gender adversely affects survival following surgery for colorectal cancer. *Br J Surg* 2003; 90(6):711-5.
41. Majek O, Gondos A, Jansen L, et al. Sex differences in colorectal cancer survival: population-based analysis of 164,996 colorectal cancer patients in Germany. *PLoS One* 2013; 8(7):e68077.
42. Micheli A, Ciampichini R, Oberaigner W, et al. The advantage of women in cancer survival: an analysis of EUROCARE-4 data. *Eur J Cancer* 2009; 45(6):1017-27.
43. van Eeghen EE, Bakker SD, van Bochove A, et al. Impact of age and comorbidity on survival in colorectal cancer. *J Gastrointest Oncol* 2015; 6(6):605-12.
44. Pule ML, Buckley E, Niyonsenga T, et al. The effects of comorbidity on colorectal cancer mortality in an Australian cancer population. *Sci Rep* 2019; 9(1):8580.
45. Guinney J, Dienstmann R, Wang X, et al. The consensus molecular subtypes of colorectal cancer. *Nat Med* 2015; 21(11):1350-6.



Chapter 4

Between-hospital variation in treatment and outcomes in stage I-III colon cancer patients

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Submitted

ABSTRACT

Introduction

Comparing treatments and outcomes across hospitals has the potential to inform best practices and identify potential for quality improvement. The study aim was to analyze differences in treatment and survival outcomes between hospitals treating patients with stage I-III colon cancer.

Methods

Patients from the MATCH study, a large multicenter study in seven hospitals in one region in the Netherlands conducted between 2007 and 2017 were included. To analyze hospital effects on recurrence free survival (RFS) and overall survival (OS), mixed effects Cox proportional hazards models were used and effects were presented as hazard ratio's (HR).

Results

Data of 1,747 patients were included. Predictors for shorter RFS included T-stage (compared to T1: T2 HR 2.35, 95%CI 1.01-5.03, T3 HR 4.48, 95%CI 1.78-11.24, T4 HR 3.51, 95%CI 3.51-23.83) and R1-resection (compared with R0, HR 2.18, 95%CI 1.01-4.70). Adjuvant chemotherapy was associated with longer RFS (HR 0.59, 95%CI 0.42-0.83). Between-hospital differences were found in RFS (p=0.036). Predictors for shorter OS included male gender (HR 1.26, 95%CI 1.06-1.50), older age (HR 1.05, 95%CI 1.05-1.07) and ASA III/IV (compared with ASA I, HR 1.57, 95%CI 1.11-2.22). Colon descendens tumors (HR 0.83, 95%CI 0.69-1.00) and adjuvant chemotherapy (HR 0.73, 95%CI 0.55-0.97) were associated with longer OS. No between-hospitals differences were found in OS (p=0.284). In stage III patients, adjuvant chemotherapy receipt (p<0.001) and RFS (p=0.013) differed between hospitals.

Conclusion

Between-hospitals in the adjuvant treatment of colon cancer exists, which, after correcting for case-mix and chance variation, translates in differences in RFS but not in OS.

INTRODUCTION

Colon cancer is one of the most common types of cancer in the Netherlands with an incidence of around 9,800 patients in 2018 [1]. Surgical resection of the primary tumor is considered the backbone of treatment for patients with stage I-III colon cancer with a 5-year survival rate between 92 and 53%[2]. For patients with lymph node involvement (stage III), standard treatment following surgical resection consists of adjuvant systemic chemotherapy. Still, clinical outcomes of individual patients with resectable tumors vary [3]. Besides patient factors, disease and treatment factors have been associated with survival [4-6]. Specific treatments, including surgical resection margins and administration of adjuvant chemotherapy in stage III patients, can have a significant effect on survival [7]. To be able to identify predictive and prognostic markers and outcome of these variables, the MATCH-study was performed [8-10]. In this prospective multicenter cohort registry, patients with stage I-III colon cancer who underwent curative intent surgery in one of seven participating hospitals in the larger region of Rotterdam in the Netherlands, were enrolled between 2007 and 2017 [10].

Data from this multicenter study, enables comparison of treatments and outcomes across the participating hospitals which has the potential to inform best practices and identify potential for quality improvement. This can be challenging because outcomes may also depend on the patient's age, preoperative condition, and disease severity and these may vary between hospitals. Such case-mix differences between hospitals, as well as chance variation related to caseload can have an effect on estimated hospital performance. Previous studies demonstrated the importance of adjusting for hospital case-mix and random variation in the comparison of outcomes [11, 12]. Random-effects regression models can be fitted to account for the fact that part of the variation in outcomes between hospitals is due to chance [13, 14]. The study aim was to analyze differences in treatment and survival outcomes in stage I-III colon cancer between the participating hospitals in a single region prospective multicenter study.

METHODS

Study population

Patients with stage I-III colon cancer who underwent curative intent surgery, enrolled in the MATCH-study between 2007 and 2017, were included in this study. The study was approved by the Erasmus MC institutional research board (MEC-2007-088) and all patients provided written informed consent.

Predictors and outcome measures

Hospitals were anonymized. Demographic variables included age and gender. Clinical variables included American Society of Anesthesiologists (ASA) score, International Union Against Cancer tumor node metastasis (TNM) classification of malignant tumors, tumor morphology, tumor differentiation grade, tumor location, and comorbidities. Treatment variables included are resection margin, operation urgency (e.g. emergency or elective resection), and adjuvant chemotherapy.

Outcomes were recurrence free survival (RFS), examined and defined as the day of surgery to the day of recurrence or last moment of follow up. Overall survival (OS), was examined and defined as the date of surgery to the day of death or last moment of follow up. Date of death was obtained from the Dutch national cancer registry.

In patients with stage III colon cancer, we examined whether patients received adjuvant chemotherapy since this is recommend in the guideline.

Statistical methods

Analyses were performed using SPSS Version 24.0 (SPSS, Inc., Chicago, IL, USA) and R version 3.6 (R Foundation, Vienna, Austria). Differences in baseline characteristics between the two treatment groups (surgery plus adjuvant chemotherapy versus surgery alone) were tested with Pearson's Chi-square analysis or Mann-Whitney U-test as appropriate.

The probability of receiving adjuvant chemotherapy was estimated using a logistic regression containing both fixed and random effects, adjusting for age, BMI, ASA score, T- and N-stage. The fixed-effects regression coefficients quantify the relation between the covariates and the outcome. The random effects estimates can be interpreted as in between-hospital variation in outcome that cannot be ascribed to fixed-effects (case-mix) or chance variation. Hence, it may reflect the hospital variation that is due to actual differences between hospitals and possibly in quality of care.

Kaplan-Meier curves were used to investigate the RFS and OS unadjusted for case-mix and were evaluated using the logrank test. After this, the RFS and OS were estimated using Cox proportional hazards models containing both fixed and random effects, and adjusted for gender, age, BMI, ASA score, location of the tumor, differentiation grade, TNM stage, tumor stage, adjuvant therapy, urgency, resection margin, and comorbidities. Details concerning the case-mix adjustment models have been described previously [12]. In both analyses, the likelihood-ratio test was used to test if between-hospital variation is present. Missing values were imputed by multiple imputation.

RESULTS

Data of 1,747 patients diagnosed with stage I-III colon cancer were included in this study. Patients with missing date of surgery were excluded (n=9). To analyze RFS, patients with recurrent disease but with missing date of recurrence, and patients without date of last follow up were omitted (n=52). To analyze OS, patients without last follow up date and date of death were excluded from the analysis (n=2).

Median age at surgery was 70 years (IQR: 64-77) and 53% of patients were male (Table 1). Differences in baseline characteristics were seen between hospitals regarding: BMI (p=0.002), ASA (p<0.001), tumor location (p=0.002), tumor differentiation grade (p<0.001), T-stage (p<0.001), tumor stage (p=0.005) and history of cerebrovascular accident (p=0.016) (Table 1). Treatment also differed between the hospitals, namely administration of adjuvant chemotherapy (p<0.001) (Table 1).

Median RFS was 46 months (IQR 21 – 65). Unadjusted RFS curves per hospital indicated differences between hospitals in outcome (p=0.036) (Figure 1a). A Cox proportional hazards model containing both fixed and random effects revealed a significant association between shorter RFS and T-stage (compared with T-stage 1, T-stage 2: HR 2.35, 95%CI 1.01-5.03, p=0.049, T-stage 3: HR 4.48, 95%CI 1.78-11.24, p=0.001, T-stage 4: HR 3.51, 95%CI 3.51-23.83, p<0.001) and R1-resection (compared with R0-resection, HR 2.18, 95%CI 1.01-4.70, p=0.049) (Table 2). Treatment with adjuvant chemotherapy was associated with longer RFS in stage III patients (HR 0.59, 95% CI 0.42-0.83, p=0.002) (Table 2). After adjustment for case-mix and chance variation, the differences in outcome between the hospitals regarding RFS remained (p=0.036) (Figure 1c and Supplementary Table 1).

The median OS was 5.2 years (IQR 3.6 – 8.2). Unadjusted OS curves of the hospitals showed significant differences between hospitals (p=0.017) (Figure 1b). A Cox proportional hazard model containing both fixed and random effects showed that independent predictors for impaired OS include male gender (HR 1.26, 95%CI 1.06-1.50, p=0.009), older age (HR per year 1.05, 95%CI 1.05-1.07, p<0.001), ASA-score III/IV (compared with ASA I, HR 1.57, 95%CI 1.11-2.22, p=0.010) and suffering lung disease(s) (HR 1.44, 95%CI 1.16-1.78, p=0.001) (Table 3). Tumors in the descending colon (HR 0.83, 95%CI 0.69-1.00, p=0.045) and treatment with adjuvant chemotherapy (HR 0.73, 95%CI 0.55-0.97, p=0.032) were associated with longer OS (Table 3). After adjustment for case-mix and chance-variation, the differences in OS between hospitals were not significant (p=0.284) (Figure 1d and Supplementary Table 1).

Table 1. Descriptive statistics in total and per hospital.

	Total	(%)	H1	(%)	H2	(%)	H3	(%)	H4	(%)	H5	(%)	H6	(%)	H7	(%)	p-value[1]
N	1,748		54		282		286		434		111		257		324		
Males	924	(53)	27		151		133		234		65		132		182		0.234
Age[2], median [IQR]	70	[64-77]	70	[61-76]	70	[65-76]	72	[65-80]	70	[62.2- 77]	70	[64-75]	69	[64- 76]	72	[64-79]	0.076
BMI																	
Underweight (BMI < 18.5)	30	(2)	0	(0)	4	(1)	7	(2)	8	(1)	2	(1)	3	(1)	6	(2)	0.013
Healthy	1,097	(63)	34	(50)	178	(50)	174	(50)	291	(50)	74	(50)	152	(50)	194	(50)	
Overweight (BMI 25)	1,070	(61)	34	(50)	174	(49)	167	(48)	284	(49)	72	(49)	150	(49)	189	(49)	
Missing	120	(7)	3		7		18		32		6		18		36		
ASA																	
I	293	(17)	10	(19)	50	(18)	50	(18)	55	(13)	17	(15)	39	(15)	72	(22)	0.004
II	1,137	(65)	26	(48)	178	(63)	185	(65)	304	(70)	82	(74)	164	(64)	198	(61)	
III	311	(18)	17	(32)	53	(19)	51	(18)	75	(17)	12	(11)	51	(20)	52	(16)	
IV	7	(0)	1	(2)	1	(0)	0	0	0	0	0	0	3	(1)	2	(1)	
Missing	164	(9)	7		63		20		2		0		30		42		
Tumor location																	
Colon ascendens	750	(43)	26	(48)	132	(47)	130	(46)	165	(38)	47	(42)	107	(42)	143	(44)	0.002
Colon transversum	119	(7)	0	(0)	17	(6)	17	(6)	35	(8)	5	(5)	27	(11)	18	(6)	
Colon descendens	855	(49)	28	(52)	129	(46)	136	(48)	223	(51)	58	(52)	122	(48)	159	(49)	
Colon NOS	24	(1)	0	(0)	4	(1)	3	(1)	11	(3)	1	(1)	1	(0)	4	(1)	

Table 1. Descriptive statistics in total and per hospital. (continued)

	Total	(%)	H1	(%)	H2	(%)	H3	(%)	H4	(%)	H5	(%)	H6	(%)	H7	(%)	p-value[1]
Morfology																	
Adenocarcinoma	1,500	(86)	42	(78)	234	(85)	246	(86)	387	(89)	94	(85)	222	(87)	275	(85)	0.106
Signet ring cell carcinoma	22	(1)	1	(2)	5	(2)	1	(0)	3	(1)	3	(3)	3	(1)	6	(2)	
Tubular adenocarcinoma	1	(0)	0	(0)	1	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	
Mucinous carcinoma	209	(12)	11	(20)	35	(13)	36	(13)	44	(10)	14	(13)	29	(11)	40	(12)	
Carcinoma NOS	4	(0)	0	(0)	0	(0)	2	(1)	0	(0)	0	(0)	1	(0)	1	(0)	
Carcinoid	3	(0)	0	(0)	1	(0)	0	(0)	0	(0)	0	(0)	1	(0)	1	(0)	
Adenosquamous carcinoma	1	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	1	(0)	
Differentiation																	
Well differentiated	43	(3)	4	(7)	12	(4)	1	(0)	5	(1)	4	(4)	5	(2)	12	(4)	0.006
Moderately differentiated	1,325	(76)	32	(59)	210	(75)	226	(79)	337	(78)	91	(82)	186	(72)	243	(75)	
Poorly or undifferentiated	196	(11)	13	(24)	31	(11)	34	(12)	43	(10)	6	(5)	22	(8)	47	(15)	
Diff. grade unknown	184	(11)	5	(9)	29	(10)	25	(9)	49	(11)	10	(9)	44	(17)	22	(7)	
T stage																	
T1	189	(11)	3	(6)	44	(16)	25	(9)	59	(14)	11	(10)	28	(11)	19	(6)	<0.001
T2	448	(26)	17	(32)	66	(23)	70	(25)	133	(31)	22	(20)	69	(27)	71	(22)	
T3	968	(55)	30	(56)	157	(56)	167	(58)	219	(51)	70	(63)	136	(53)	189	(58)	
T4	143	(8)	4	(7)	15	(5)	24	(8)	23	(5)	8	(7)	24	(9)	45	(14)	
Missing	2	(0)	0		0		0		0		0		2		0		

Table 1. Descriptive statistics in total and per hospital. (continued)

	Total	(%)	H1	(%)	H2	(%)	H3	(%)	H4	(%)	H5	(%)	H6	(%)	H7	(%)	p-value[1]
N stage																	
N0	1,194	(68)	37	(69)	203	(72)	199	(70)	305	(70)	78	(70)	175	(68)	197	(61)	0.234
N1	374	(21)	12	(22)	61	(22)	58	(20)	82	(19)	22	(20)	56	(22)	83	(26)	
N2	180	(10)	5	(9)	18	(6)	29	(10)	47	(11)	11	(10)	26	(10)	44	(14)	
Missing	20	(1)	0		7		2		3		1		2		5		
Tumor stage																	
I	531	(30)	14	(26)	92	(33)	80	(28)	158	(36)	30	(27)	84	(33)	73	(23)	0.008
II	663	(38)	23	(43)	111	(39)	119	(42)	147	(34)	48	(43)	92	(36)	123	(38)	
III	554	(32)	17	(32)	79	(28)	87	(30)	129	(30)	33	(30)	81	(32)	128	(40)	
Adjuvant chemotherapy[3]	320	(58)	9	(53)	24	(30)	64	(74)	92	(71)	24	(73)	38	(47)	69	(54)	<0.001
Time gap between surgery and chemo																	
Start chemo 8 weeks	264	(83)	6	(67)	14	(58)	56	(88)	79	(86)	20	(83)	35	(92)	54	(78)	<0.001
Start chemo 8 weeks	56	(17)	3	(33)	10	(42)	8	(13)	13	(14)	4	(17)	3	(8)	15	(22)	
Surgery timing																	
No emergency	1,730	(99)	54	(100)	280	(99)	283	(99)	432	(100)	110	(99)	250	(97)	321	(99)	0.828
Emergency	11	(1)	0	(0)	1	(0)	2	(1)	2	(1)	1	(1)	4	(2)	1	(0)	
Planned	2	(0)	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)	1	(0)	1	(0)	
Urgent	5	(0)	0	(0)	1	(0)	1	(0)	0	(0)	0	(0)	2	(1)	1	(0)	
Missing	48	(3)	1		8		3		11		0		18		7		
Resection margin																	
R0 resection	1,632	(93)	53	(98)	273	(97)	276	(97)	423	(98)	76	(69)	227	(88)	304	(94)	0.281
R1 resection	15	(1)	1	(2)	3	(1)	1	(0)	4	(1)	0	(0)	5	(2)	1	(0)	
Resection unknown	101	(6)	0	(0)	6	(2)	9	(3)	7	(2)	35	(32)	25	(10)	19	(6)	
Comorbidities																	

Table 1. Descriptive statistics in total and per hospital. (continued)

	Total	(%)	H1	(%)	H2	(%)	H3	(%)	H4	(%)	H5	(%)	H6	(%)	H7	(%)	p-value[1]
Lung disease	243	(14)	10	(14)	41	(12)	33	(11)	56	(12)	14	(11)	41	(13)	48	(14)	0.626
Cardiovascular disease	558	(32)	19	(26)	97	(29)	92	(30)	135	(29)	29	(23)	91	(28)	95	(27)	0.549
Hypertension	695	(40)	26	(36)	114	(34)	109	(35)	174	(37)	45	(35)	99	(31)	128	(36)	0.903
Cerebrovascular accident	92	(5)	4	(6)	14	(4)	15	(5)	12	(3)	8	(6)	24	(7)	15	(4)	0.014
Liver disease	27	(2)	2	(3)	7	(2)	1	(0)	4	(1)	3	(2)	3	(1)	7	(2)	0.114
Kidney disease	64	(4)	1	(1)	13	(4)	12	(4)	15	(3)	3	(2)	11	(3)	9	(3)	0.857
Diabetes	314	(18)	11	(15)	51	(15)	46	(15)	76	(16)	25	(20)	54	(17)	51	(14)	0.565
1 comorbidities missing	13	(1)	0		1		0		0		1		1		10		

[1]p-values for continuous variable (age) are obtained using the Mann-Whitney U-test, other p-values are obtained using the Chi-square test.
[2]Instead of frequency and percentage, the median and IQR are presented for the variable age.
[3]Percentages from stage III patients (n=558)
Abbreviations: NOS: not otherwise specified

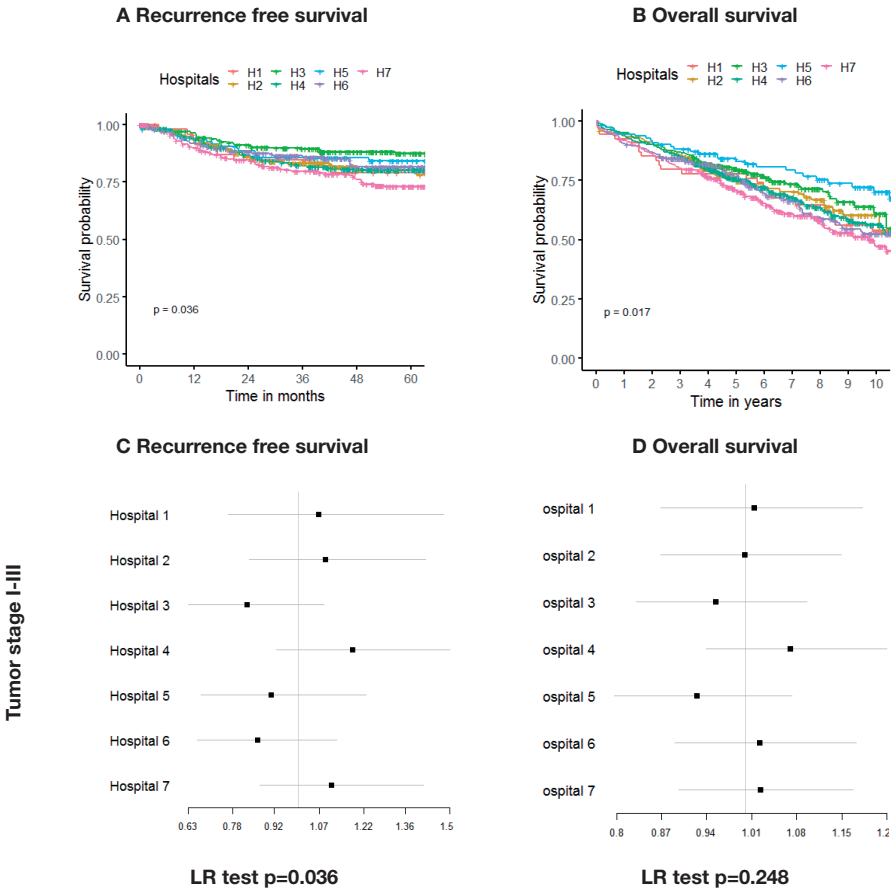

Figure 1a. Unadjusted RFS curves in months per hospital. **1b.** Unadjusted OS curves in years per hospital. **1c.** Adjusted random effects in hazard ratios per hospital on RFS. **1d.** Adjusted random effects in hazard ratios per hospital on OS.
X-axis = Hazard ratios; Abbreviations: LR-test = likelihood-ratio test for differences between hospitals

Table 2. Cox proportional hazards model containing both fixed and random effects of RFS in months and OS in years

	Recurrence free survival			Overall survival		
	HR	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Sex (female: ref)	1.00	(0.78 - 1.27)	0.980	1.26	(1.06 - 1.50)	0.009
Age	1.01	(0.99 - 1.02)	0.250	1.06	(1.05 - 1.07)	<0.001
BMI						
Underweight (BMI < 18)	0.74	(0.30 - 1.84)	0.520	1.78	(1.06 - 3.01)	0.030
Healthy: ref						
Overweight (BMI 25)	0.84	(0.66 - 1.07)	0.160	0.86	(0.72 - 1.03)	0.110
ASA						
I: ref						
II	0.81	(0.58 - 1.11)	0.190	0.91	(0.68 - 1.22)	0.530
III-IV	0.85	(0.53 - 1.36)	0.500	1.57	(1.11 - 2.22)	0.010
Tumor location						
Colon ascendens: ref						
Colon transversum	0.73	(0.42 - 1.29)	0.280	1.14	(0.82 - 1.59)	0.420
Colon descendens	1.04	(0.81 - 1.34)	0.750	0.83	(0.69 - 1.00)	0.045
Colon NOS	0.72	(0.18 - 2.95)	0.650	1.62	(0.87 - 3.01)	0.120
Differentiation grade						
Well differentiated: ref						
Moderately differentiated	1.23	(0.53 - 2.82)	0.630	0.70	(0.44 - 1.10)	0.120
Poorly or undifferentiated	1.37	(0.57 - 3.28)	0.480	0.94	(0.57 - 1.54)	0.810
Diff. grade unknown	1.45	(0.60 - 3.52)	0.410	0.79	(0.47 - 1.31)	0.350
T-stage						
T1: ref						
T2	2.25	(1.01 - 5.03)	0.049	0.94	(0.65 - 1.34)	0.720
T3	4.48	(1.78 - 11.24)	0.001	1.03	(0.64 - 1.67)	0.900
T4	9.14	(3.51 - 23.83)	<0.001	1.33	(0.77 - 2.30)	0.310
N-stage						
N0: ref						
N1	0.30	(0.05 - 2.01)	0.220	1.69	(0.02 - 124.87)	0.810
N2	0.60	(0.09 - 3.98)	0.600	2.95	(0.04 - 218.83)	0.620
Tumor stage						
I: ref						
II	0.78	(0.39 - 1.57)	0.490	0.95	(0.62 - 1.47)	0.820
III	7.17	(0.95 - 53.83)	0.056	1.03	(0.01 - 77.57)	0.990
Adjuvant chemotherapy	0.59	(0.42 - 0.83)	0.002	0.73	(0.55 - 0.97)	0.032
Surgery timing						
Elective procedure: ref						
Emergency surgery	1.39	(0.56 - 3.47)	0.480	1.33	(0.62 - 2.87)	0.470

Table 2. Cox proportional hazards model containing both fixed and random effects of RFS in months and OS in years (continued)

	Recurrence free survival			Overall survival		
	HR	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Resection margin						
R0: ref						
R1 resection	2.18	(1.01 - 4.70)	0.046	1.62	(0.80 - 3.32)	0.180
Comorbidities						
Lung disease	0.85	(0.59 - 1.23)	0.400	1.44	(1.16 - 1.78)	0.001
Cardiovascular disease	1.05	(0.79 - 1.39)	0.750	1.04	(0.86 - 1.26)	0.680
Hypertension	0.74	(0.57 - 0.97)	0.029	0.83	(0.70 - 0.99)	0.042
Cerebrovasculair accident	1.05	(0.59 - 1.86)	0.880	1.19	(0.87 - 1.63)	0.280
Liver disease	1.75	(0.77 - 4.00)	0.180	1.89	(1.03 - 3.47)	0.040
Kidney disease	0.77	(0.37 - 1.60)	0.480	1.04	(0.69 - 1.58)	0.840
Diabetes	0.96	(0.69 - 1.34)	0.810	1.03	(0.83 - 1.29)	0.760

Abbreviations: HR, Hazard ratio; CI, confidence interval Adjuvant chemotherapy in stage III colon cancer patients

In total, 320 of the 558 patients (57.3%) with stage III colon cancer received adjuvant chemotherapy. Older patients and underweight patients were less likely to receive adjuvant chemotherapy (OR 0.89 per year, 95% CI 0.86-0.91, P<0.001 and OR 0.19, 95% CI 0.04-0.96, P=0.040, respectively) (Table 3).

The differences between hospitals regarding administration of adjuvant chemotherapy remained when correcting for the case-mix variables and chance-variation (p<0.001) (Figure 2 and Supplementary Table 2).

The unadjusted survival analyses in stage III patients showed differences in RFS, but not in OS between hospitals (p=0.009 and p=0.17, respectively) (Figure 3a+b). Independent predictors for impaired RFS and OS in stage III patients were comparable to all patients (Supplementary Table 3). When correcting for case-mix and chance-variation, the difference in RFS between the hospitals remained (p=0.013) (Figure 3c+d and Supplementary Table 1).

Table 3. Logistic regression analyses containing both fixed and random effects on the probability of receiving adjuvant chemotherapy

	Adjuvant chemotherapy		
	OR	**95% CI**	**p-value**
Age	0.89	(0.86 - 0.91)	<0.001
BMI			
Underweight (BMI < 18)	0.19	(0.04 - 0.96)	0.040
Healthy: ref			
Overweight (BMI 25)	1.28	(0.83 - 1.98)	0.260
ASA			
ASA I: ref			
ASA II	1.04	(0.59 - 1.82)	0.890
ASA III/IV	0.56	(0.28 - 1.13)	0.100
T-stage			
T1: ref			
T2	0.90	(0.29 - 2.81)	0.850
T3	1.21	(0.42 - 3.47)	0.720
T4	1.37	(0.43 - 4.41)	0.600
N-stage			
N1: ref			
N2	1.40	(0.89 - 2.20)	0.150

Abbreviations: OR, odds ratio; CI, confidence interval

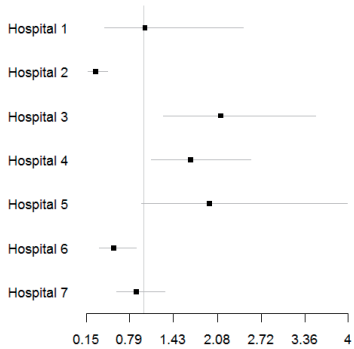

Figure 2. The random effects estimates in odds ratios which can be interpreted as in between-hospital variation in administering adjuvant chemotherapy that cannot be ascribed to case-mix or chance variation.
X-axis = Odds ratios; Abbreviations: LR-test = likelihood-ratio test for differences between hospitals

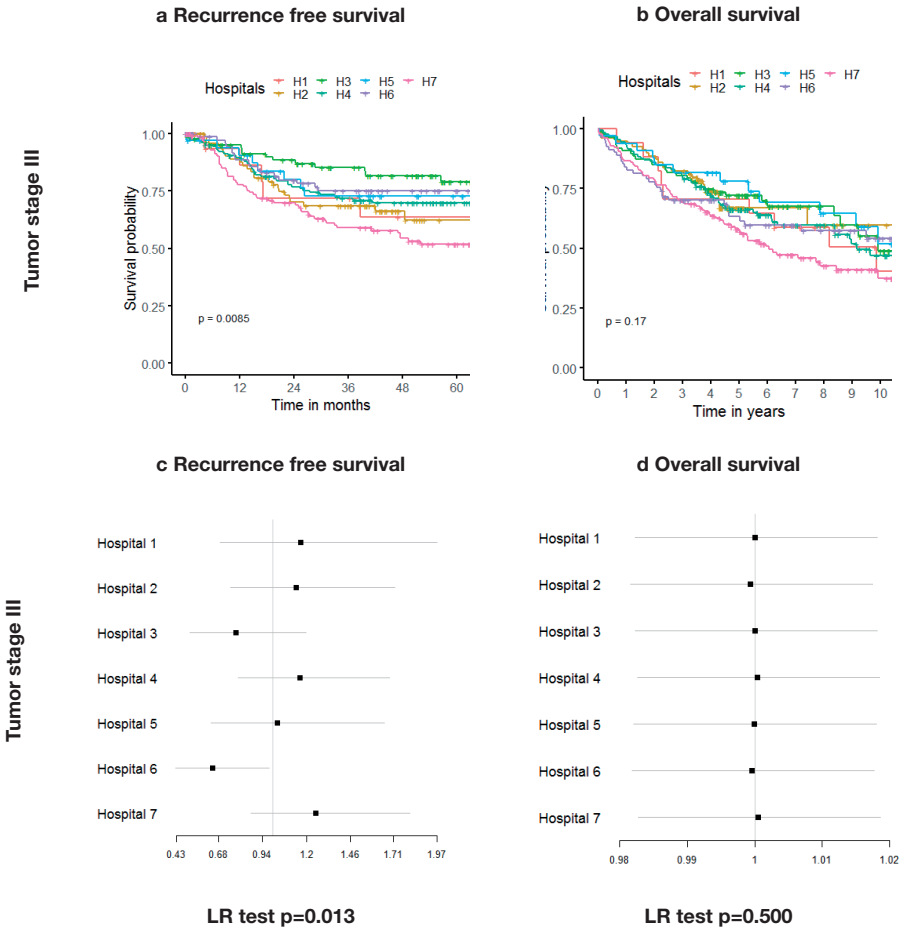

Figure 3a. Unadjusted RFS curves in months per hospital. **3b.** Unadjusted OS curves in years per hospital. **3c.** Adjusted random effects in hazard ratios per hospital on RFS in stage III patients. **3d.** Adjusted random effects in hazard ratios per hospital on OS in stage III patients.
X-axis = Hazard ratios; Abbreviations: LR-test = likelihood-ratio test

DISCUSSION

Our study shows a detailed, descriptive overview of nearly 1,800 colon cancer patients who were included in a large, prospective single region multicenter study in the Netherlands. For the entire study cohort, differences in RFS but not in OS were present between hospitals, even when correcting for case-mix and change variation. In stage III patients, administration of adjuvant chemotherapy differed substantially between hospitals, even when correcting for case-mix and chance variation. Survival analyses in these patients showed significant RFS, but no OS, differences between hospitals.

The independent predictors for impaired RFS (high T-stage, R1 resection and lack of receipt of adjuvant chemotherapy) and OS (male gender, older age, ASA-score III/IV, lung diseases, and lack of receipt of adjuvant chemotherapy) found in this study are in line with previous literature [15-21]. A trend between low BMI and impaired OS was observed (p=0.056), which is in line with multiple studies showing that colon cancer patients with low BMI have a worse survival [15, 16, 22]. Our results also show that patients with a low BMI are less likely to receive adjuvant chemotherapy thereby suggesting an additional effect impaired survival for these patients. Two recent pooled analyses in ovarian cancer and metastasized colorectal cancer respectively, showed that low BMI was significantly associated with more toxicity and preterm termination of the chemotherapy [23, 24]. Therefore, the administration of chemotherapy in underweight cancer patients should be a shared decision taking these findings into consideration. Furthermore, previous research shows that low skeletal muscle mass and density is also predictive for colon cancer outcomes and may be better predictors of surgical outcomes than BMI [25-27]. Addition of sarcopenia screening for patients undergoing oncological treatment (chemotherapy or surgery) could be a next step to improve care of colon cancer patients.

With the availability of the MATCH data, recurrence and mortality rates between hospitals can be studied in a well-defined population within one region, inhabited by 1.2 million people to identify potential differences in hospital performance, while taking into account potential biases [28, 29]. In this study, case-mix variables were significantly different across the hospitals, especially the distribution of disease specific and survival determinative factors, such as tumor stage and ASA score. After correcting for these factors, the seven hospitals showed similar overall survival outcomes indicating that overall patient care is well comparable.

Interestingly, in contrast to OS, RFS remained significantly different between hospitals after correction for relevant factors. This is in line with literature suggesting that more specific outcomes, such as RFS, are more sensitive as a measure of quality of hospital care than general outcomes such as OS [11]. The differences we found in RFS may reflect actual differences between hospitals in (quality of) care and/or relevant confounders that were not considered but do impact clinical practice or long-term outcome. One of these differences observed in the current study was the significant difference regarding administration of adjuvant chemotherapy. These findings are in line with previous studies demonstrating significant practice variation in the adjuvant treatment of colon cancer on hospital level [30, 31]. In the most recent study by Keikes et al., the variation was predominantly found in high-risk stage II colon cancer patients for which guidelines leaves some room for variation [30]. We observed these differences in stage III patients, for which the indication of adjuvant chemotherapy is unambiguous. Similarly to their findings, we found age to be the strongest predictor for non-receipt of adjuvant treatment despite the fact that multiple studies have already demonstrated safety and efficacy of adjuvant chemotherapy in elderly patients [32-34]. Non-receipt of adjuvant therapy may have been entirely

appropriate given circumstances of individual patients. For instance, frail, old and underweight patients are more likely to die from non-cancerous diseases and for them, chemotherapy could even be more harmful than beneficial. The similar OS between hospitals could therefore imply that the choice whether or not to administer chemotherapy was well considered. On the other hand, the survival benefit from adjuvant chemotherapy for young, otherwise fit, patients is absolute. For those patients, the risk of recurrence and possible causal death can be greater than non-cancerous mortality. Unfortunately, our study is limited by lacking data on cancer-specific-survival which could give additional information regarding the difference in survival.

Since death is the most undesirable outcome for patients, mortality has strong face validity as a measure of hospital care. However, even though outcome indicators such as survival are highly relevant for patients and provide information on aspects of delivered care, they are confounded by factors unrelated to quality of care. Therefore, it is important to also analyze process indicators. Measuring quality of care and reflecting on outcome differences can lead to improvement by learning from best practice. Despite the tendency to prefer outcome indicators over process indicators, opportunities for improvement are hard to identify based on outcomes indicators alone. Process indicators, such as adjuvant treatment and timing, however, are generally evidence-based and are directly attributable to delivered care. Plus, they are generally less prone to case-mix variability. On the other hand, process indicators also just represent what can be measured and may be less meaningful to patients.

The strength of this study is that we analyze outcome as well as process indicators in a large prospective multicenter study. Underlying reasons for treatment strategies that are non-adherent to the current guidelines should be reported in patient records in order to learn from these outcomes. The differences in guideline adherence regarding adjuvant treatment warrants for new strategies to improve awareness on guideline adherence and implementation. Proper documentation and comparing strategies and analyzing the differences can help in identifying best practice. The evaluation of guideline adherence and implementation can be facilitated by hospital auditing, of which the positive effect on patient outcomes has been demonstrated in previous studies [35, 36].

This study demonstrates that even after adjusting for case-mix variables and chance variation, between-hospital variation in treatment and outcome in stage I-III colon cancer patients is present. With relatively equal surgical outcomes, such as resection margins, improvements can be made in appropriate administration of chemotherapy which in turn may improve oncologic outcomes. Reflecting on process indicators and outcome differences can lead to improvement by learning from best practice.

REFERENCES

1. Nederlandse Kankerregistratie (NKR), IKNL.
2. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, et al. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. *Eur J Cancer* 2013; 49(6):1374-403.
3. Dienstmann R, Mason MJ, Sinicrope FA, et al. Prediction of overall survival in stage II and III colon cancer beyond TNM system: a retrospective, pooled biomarker study. *Ann Oncol* 2017; 28(5):1023-1031.
4. Karahalios A, English DR, Simpson JA. Weight change and risk of colorectal cancer: a systematic review and meta-analysis. *Am J Epidemiol* 2015; 181(11):832-45.
5. Lauby-Secretan B, Scoccianti C, Loomis D, et al. Body Fatness and Cancer--Viewpoint of the IARC Working Group. *N Engl J Med* 2016; 375(8):794-8.
6. Yuhara H, Steinmaus C, Cohen SE, et al. Is diabetes mellitus an independent risk factor for colon cancer and rectal cancer? *Am J Gastroenterol* 2011; 106(11):1911-21; quiz 1922.
7. Gao P, Huang XZ, Song YX, et al. Impact of timing of adjuvant chemotherapy on survival in stage III colon cancer: a population-based study. *BMC Cancer* 2018; 18(1):234.
8. Coebergh van den Braak RRJ, Lalmahomed ZS, Buttner S, et al. Nonphysician Clinicians in the Follow-Up of Resected Patients with Colorectal Cancer. *Dig Dis* 2018; 36(1):17-25.
9. Lalmahomed ZS, Coebergh van den Braak RRJ, Oomen MHA, et al. Multicenter fresh frozen tissue sampling in colorectal cancer: does the quality meet the standards for state of the art biomarker research? *Cell Tissue Bank* 2017; 18(3):425-431.
10. Kloosterman WP, Coebergh van den Braak RRJ, Pieterse M, et al. A systematic analysis of oncogenic gene fusions in primary colon cancer. *Cancer Res* 2017.
11. Fischer C, Lingsma HF, van Leersum N, et al. Comparing colon cancer outcomes: The impact of low hospital case volume and case-mix adjustment. *Eur J Surg Oncol* 2015; 41(8):1045-53.
12. Kolfschoten NE, Marang van de Mheen PJ, Gooiker GA, et al. Variation in case-mix between hospitals treating colorectal cancer patients in the Netherlands. *Eur J Surg Oncol* 2011; 37(11):956-63.
13. Dimick JB, Ghaferi AA, Osborne NH, et al. Reliability adjustment for reporting hospital outcomes with surgery. *Ann Surg* 2012; 255(4):703-7.
14. Dimick JB, Staiger DO, Birkmeyer JD. Ranking hospitals on surgical mortality: the importance of reliability adjustment. *Health Serv Res* 2010; 45(6 Pt 1):1614-29.
15. Shahjehan F, Merchea A, Cochuyt JJ, et al. Body Mass Index and Long-Term Outcomes in Patients With Colorectal Cancer. *Front Oncol* 2018; 8:620.
16. Shibakita M, Yoshimura H, Tachibana M, et al. Body mass index influences long-term outcome in patients with colorectal cancer. *Hepatogastroenterology* 2010; 57(97):62-9.
17. Majek O, Gondos A, Jansen L, et al. Sex differences in colorectal cancer survival: population-based analysis of 164,996 colorectal cancer patients in Germany. *PLoS One* 2013; 8(7):e68077.
18. Bartolini I, Ringressi MN, Melli F, et al. Analysis of Prognostic Factors for Resected Synchronous and Metachronous Liver Metastases from Colorectal Cancer. *Gastroenterol Res Pract* 2018; 2018:5353727.
19. Wilson RJT, Yates DRA, Walkington JP, et al. Ventilatory inefficiency adversely affects outcomes and longer-term survival after planned colorectal cancer surgery. *Br J Anaesth* 2019; 123(2):238-245.

20. Lim DR, Kuk JK, Kim T, et al. Comparison of oncological outcomes of right-sided colon cancer versus left-sided colon cancer after curative resection: Which side is better outcome? *Medicine (Baltimore)* 2017; 96(42):e8241.
21. Zafar SN, Hu CY, Snyder RA, et al. Predicting Risk of Recurrence After Colorectal Cancer Surgery in the United States: An Analysis of a Special Commission on Cancer National Study. *Ann Surg Oncol* 2020; 27(8):2740-2749.
22. Kaneko M, Sasaki S, Ozaki K, et al. Underweight status predicts a poor prognosis in elderly patients with colorectal cancer. *Mol Clin Oncol* 2016; 5(3):289-294.
23. Grabowski JP, Richter R, Rittmeister H, et al. Impact of Body Mass Index (BMI) on Chemotherapy-associated Toxicity in Ovarian Cancer Patients. A Pooled Analysis of the North-Eastern German Society of Gynecological Oncology (NOGGO) Databank on 1,213 Patients. *Anticancer Res* 2018; 38(10):5853-5858.
24. Abdel-Rahman O. Effect of Body Mass Index on 5-FU-Based Chemotherapy Toxicity and Efficacy Among Patients With Metastatic Colorectal Cancer; A Pooled Analysis of 5 Randomized Trials. *Clin Colorectal Cancer* 2019; 18(4):e385-e393.
25. Jochum SB, Kistner M, Wood EH, et al. Is sarcopenia a better predictor of complications than body mass index? Sarcopenia and surgical outcomes in patients with rectal cancer. *Colorectal Dis* 2019; 21(12):1372-1378.
26. van Vugt JLA, Coebergh van den Braak RRJ, Lalmahomed ZS, et al. Impact of low skeletal muscle mass and density on short and long-term outcome after resection of stage I-III colorectal cancer. *Eur J Surg Oncol* 2018; 44(9):1354-1360.
27. Vergara-Fernandez O, Trejo-Avila M, Salgado-Nesme N. Sarcopenia in patients with colorectal cancer: A comprehensive review. *World J Clin Cases* 2020; 8(7):1188-1202.
28. Lingsma HF, Steyerberg EW, Eijkemans MJ, et al. Comparing and ranking hospitals based on outcome: results from The Netherlands Stroke Survey. *QJM* 2010; 103(2):99-108.
29. Henneman D, van Bommel AC, Snijders A, et al. Ranking and rankability of hospital postoperative mortality rates in colorectal cancer surgery. *Ann Surg* 2014; 259(5):844-9.
30. Keikes L, Koopman M, Lemmens V, et al. Practice Variation in the Adjuvant Treatment of Colon Cancer in the Netherlands: A Population-based Study. *Anticancer Res* 2020; 40(8):4331-4341.
31. van Steenbergen LN, Rutten HJT, Creemers GJ, et al. Large age and hospital-dependent variation in administration of adjuvant chemotherapy for stage III colon cancer in southern Netherlands. *Ann Oncol* 2010; 21(6):1273-1278.
32. Lemmens VE, van Halteren AH, Janssen-Heijnen ML, et al. Adjuvant treatment for elderly patients with stage III colon cancer in the southern Netherlands is affected by socioeconomic status, gender, and comorbidity. *Ann Oncol* 2005; 16(5):767-72.
33. McCleary NJ, Meyerhardt JA, Green E, et al. Impact of age on the efficacy of newer adjuvant therapies in patients with stage II/III colon cancer: findings from the ACCENT database. *J Clin Oncol* 2013; 31(20):2600-6.
34. Sargent DJ, Goldberg RM, Jacobson SD, et al. A pooled analysis of adjuvant chemotherapy for resected colon cancer in elderly patients. *N Engl J Med* 2001; 345(15):1091-7.
35. Iversen LH, Green A, Ingeholm P, et al. Improved survival of colorectal cancer in Denmark during 2001-2012 - The efforts of several national initiatives. *Acta Oncol* 2016; 55 Suppl 2:10-23.
36. Coebergh van den Braak RRJ, van Rijssen LB, van Kleef JJ, et al. Nationwide comprehensive gastro-intestinal cancer cohorts: the 3P initiative. *Acta Oncol* 2018; 57(2):195-202.

Supplementary Data

4

Supplementary Table 1. Hospital effect on recurrence free and overall survival

	RFS tumor stage I-III		OS tumor stage I-III		RFS tumor stage III		OS tumor stage III	
	HR	**95% CI**	**HR**	**95% CI**	**HR**	**95% CI**	**HR**	**95% CI**
Hospital 1	1.07	(0.77 - 1.49)	1.01	(0.87 - 1.18)	1.16	(0.70 - 1.92)	1.00	(0.98- 1.02)
Hospital 2	1.09	(0.84 - 1.43)	1.00	(0.87 - 1.15)	1.12	(0.75 - 1.67)	0.99	(0.98- 1.02)
Hospital 3	0.83	(0.63 - 1.09)	0.95	(0.83 - 1.10)	0.80	(0.53 - 1.21)	1.00	(0.98- 1.02)
Hospital 4	1.18	(0.93 - 1.51)	1.07	(0.94 - 1.22)	1.13	(0.78 - 1.62)	1.00	(0.98- 1.02)
Hospital 5	0.91	(0.67 - 1.23)	0.92	(0.80 - 1.07)	1.02	(0.65 - 1.62)	0.99	(0.98- 1.02)
Hospital 6	0.86	(0.66 - 1.13)	1.02	(0.89 - 1.17)	0.67	(0.45 - 0.99)	0.99	(0.98- 1.02)
Hospital 7	1.11	(0.87 - 1.42)	1.02	(0.90 - 1.17)	1.25	(0.88 - 1.77)	1.00	(0.98- 1.02)

Abbreviations: HR, hazard ratio; CI, confidence interval

Supplementary Table 2. Hospital effect on treatment adherence

	Adjuvant chemotherapy	
	OR	**95% CI**
Hospital 1	1.03	(0.43- 2.51)
Hospital 2	0.29	(0.18- 0.48)
Hospital 3	2.16	(1.30- 3.60)
Hospital 4	1.69	(1.11- 2.59)
Hospital 5	1.96	(0.96- 3.99)
Hospital 6	0.54	(0.34- 0.87)
Hospital 7	0.83	(0.56- 1.23)

Abbreviations: OR, odds ratio; CI, confidence interval

Supplementary Table 3. Cox proportional hazards model containing both fixed and random effects of recurrence free survival in months and overall survival in years for only stage III patients

	Recurrence free survival			Overall survival		
	HR	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Sex (female: ref)	0.94	(0.68 - 1.32)	0.740	1.46	(1.09 - 1.95)	0.010
Age	1.00	(0.98 - 1.02)	0.890	1.02	(1.00 - 1.03)	0.035
BMI						
Underweight (BMI < 18)	1.25	(0.43 - 3.65)	0.680	2.76	(1.30 - 5.89)	0.009
Healthy: ref						
Overweight (BMI 25)	0.99	(0.70 - 1.41)	0.970	0.97	(0.72 - 1.31)	0.860
ASA						
I: ref						
II	0.75	(0.49 - 1.15)	0.190	0.73	(0.50 - 1.08)	0.120
III-IV	1.00	(0.55 - 1.84)	0.990	1.35	(0.83 - 2.19)	0.220
Tumor location						
Colon ascendens: ref						
Colon transversum	1.02	(0.47 - 2.22)	0.950	1.45	(0.80 - 2.63)	0.220
Colon descendens	0.87	(0.61 - 1.22)	0.410	0.75	(0.56 - 1.01)	0.060
Colon NOS	0.79	(0.10 - 5.93)	0.820	2.93	(0.88 - 9.79)	0.081
Differentiation grade						
Well differentiated: ref						
Moderately differentiated	1.03	(0.31 - 3.48)	0.960	0.64	(0.23 - 1.79)	0.400
Poorly or undifferentiated	1.17	(0.34 - 4.06)	0.800	0.86	(0.30 - 2.44)	0.770
Diff. grade unknown	1.34	(0.37 - 4.90)	0.650	0.85	(0.29 - 2.52)	0.770
T-stage						
T1: ref						
T2	3.30	(0.42 - 25.72)	0.260	0.83	(0.36 - 1.94)	0.670
T3	6.20	(0.85 - 45.24)	0.072	0.95	(0.43 - 2.1)	0.900
T4	11.23	(1.49 - 84.54)	0.019	1.36	(0.57 - 3.22)	0.490
N-stage						
N0: ref						
N1	1.98	(1.43 - 2.76)	<0.001	1.98	(1.48 - 2.64)	<0.001
Adjuvant chemotherapy	0.57	(0.39 - 0.83)	0.003	0.49	(0.36 - 0.67)	<0.001
Surgery timing						
Elective procedure: ref						
Emergency surgery	1.73	(0.61 - 4.92)	0.310	0.59	(0.14 - 2.47)	0.470
Resection margin						
R0: ref						
R1 resection	2.97	(1.17 - 7.53)	0.022	2.04	(0.87 - 4.79)	0.100

Supplementary Table 3. Cox proportional hazards model containing both fixed and random effects of recurrence free survival in months and overall survival in years for only stage III patients (continued)

	Recurrence free survival			Overall survival		
	HR	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Comorbidities						
Lung disease	0.82	(0.49 - 1.37)	0.450	1.43	(1.00 - 2.04)	0.051
Cardiovascular disease	0.98	(0.66 - 1.45)	0.900	1.10	(0.80 - 1.51)	0.550
Hypertension	0.87	(0.60 - 1.25)	0.450	0.88	(0.65 - 1.19)	0.410
Cerebrovasculair accident	1.39	(0.64 - 3.02)	0.400	0.74	(0.37 - 1.47)	0.380
Liver disease	2.13	(0.75 - 6.11)	0.160	2.28	(0.94 - 5.53)	0.067
Kidney disease	0.89	(0.38 - 2.10)	0.800	1.05	(0.52 - 2.12)	0.900
Diabetes	0.77	(0.49 - 1.22)	0.270	0.86	(0.61 - 1.22)	0.400

Abbreviations: HR, Hazard ratio; CI, confidence interval

Chapter 5

Daily practice in guideline adherence to adjuvant chemotherapy in stage III colon cancer and predictors of outcome

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ABSTRACT

Introduction

Although guidelines recommend adjuvant chemotherapy for stage III colon cancer patients, many patients do not receive adjuvant chemotherapy. The aim of this study was to identify reasons for guideline non-adherence and assess the effect on patient outcomes in a multicenter cohort of stage III colon cancer patients who received surgery plus adjuvant chemotherapy or surgery alone.

Methods

Patients who underwent surgery between 2007 and 2017 were included. Reasons for non-adherence were determined. Propensity score analyses with inverse probability weighting were performed to adjust for confounding factors. Cox proportional hazards regression and risk stratified analyses were performed to assess the association of guideline adherence and other potential predictors with recurrence free survival (RFS).

Results

Data of 575 patients were included of whom 61% received adjuvant chemotherapy. In 87 of 222 patients (39%) who did not receive adjuvant chemotherapy, no reason was documented. Only age was predictive for receiving chemotherapy. Patients who received adjuvant chemotherapy had longer RFS (HR 0.42, 95%CI 0.29–0.62, p<0.001). High T- and N-stage were associated with poorer RFS HR 2.0 (95%CI 1.58–2.71, p<0.001) and HR 2.19 (95%CI 1.60-2.99, p<0.001) respectively. Risk groups were identified with distinct prognosis and treatment effect and a nomogram is presented to visualize individualized RFS differences.

Conclusion

This study shows considerable variation in guideline adherence to adjuvant chemotherapy and poor documentation on reasons for non-adherence. Optimizing adherence and gaining insight in reasons for non-adherence is advocated as this can lead to significant RFS benefit, especially in patients with high T-and N-stage tumors.

INTRODUCTION

Colon cancer is one of the most common types of cancer in the Netherlands with an incidence of around 9,800 patients in 2018 [1]. Surgical resection of the primary tumor is considered the backbone of treatment for patients with stage I-III colon cancer with a 5-year survival rate between 92 and 53% [2]. For patients with lymph node involvement (stage III), standard treatment following surgical resection consists of adjuvant systemic chemotherapy in either a doublet schedule with oxaliplatin plus fluoropyrimidine or as monotherapy with fluoropyrimidine. Numerous studies have confirmed the survival benefit of this adjuvant treatment [3-6] and the IDEA trial recently even showed non-inferiority of 3 months versus 6 months oxaliplatin-based adjuvant therapy combined with capecitabine [7]. While there is no age limit for the administration of adjuvant chemotherapy, the added value of oxaliplatin in elderly people (> 70 years) is debatable [8, 9]. Although evidence-based guidelines recommend adjuvant chemotherapy, literature suggests that many eligible patients do not receive adjuvant chemotherapy [3, 10-14], for various reasons. The underlying reasons for this omission may vary strongly and include recommendation against the use of adjuvant therapy by the attending physician, or refusal by the patient. The high proportion of treatment decisions in which guidelines are not adhered to indicates that shared decision-making and patient specific choices are central to daily practice and require a solid foundation. In the current era of personalized treatment, identification of the impact of these decisions is of utmost importance.

We performed a study to assess daily practice regarding guideline adherence to administration of adjuvant chemotherapy and identify reasons for guideline non-adherence in a multicenter cohort of stage III colon cancer patients who received surgery plus adjuvant chemotherapy or who were treated with surgery alone.

In addition, we evaluated the outcome of adjuvant treatment on recurrence free survival (RFS). To capture treatment effect of adjuvant chemotherapy on recurrence, propensity score methods, including inverse probability of treatment weighting (IPW) were used to minimize confounding effects [15]. A risk stratified analysis of treatment effect was conducted to identify subgroups of patients having different benefit from adjuvant chemotherapy [16-19]. These analyses were used to create a nomogram for treatment effect on RFS in patients with stage III colon cancer to aid shared decision making.

METHODS

Study population

We analyzed data of patients with stage III colon cancer who underwent curative surgery and had been enrolled in the MATCH-study, a prospective multicenter cohort study including patients with stage I-III colorectal cancer from 2007 until December 2017 in seven hospitals in the region of Rotterdam, the Netherlands.[20]

The rationale of the MATCH study was to obtain fresh frozen tissue samples with matched clinical data to identify subtypes of colorectal cancer, related prognostic markers and outcome of treatment [21]. The MATCH study was approved by the Erasmus MC medical ethics review board (MEC-2007-088) and all patients provided written informed consent to use their data.

Study parameters

Patients characteristics and treatment variables were retrieved from the prospective database of the MATCH study. Patients' medical records were reviewed retrospectively to elicit reasons why the treating physician(s) had not recommended adjuvant chemotherapy, or why patients had refused chemotherapy despite medical recommendation. To establish possible associations with patient survival, we categorized the reasons for non-adherence in four groups; patients' choice, doctors' recommendation, shared, and no documentation.

Outcome measures

RFS was calculated from the day of surgery to the day of recurrence or last moment of follow up. OS, defined as all-cause mortality, was calculated from the date of surgery to the day of death or last moment of follow up. Date of death was obtained from the Netherlands national cancer registry. Data of patients who had died within 3 months following surgery were excluded from survival analyses.

Statistical methods

Descriptive statistics and multivariable analyses were performed using SPSS Version 24.0 (SPSS, Inc., Chicago, IL, USA) and R version 3.6 (R Foundation, Vienna, Austria). Differences in baseline characteristics between the two treatment groups, surgery plus adjuvant chemotherapy versus surgery alone, were tested with Pearson's Chi-square analysis or Mann-Whitney U-test as appropriate. The effect of adjuvant chemotherapy on RFS was analyzed using Cox proportional hazards regression models. To adjust for measured confounding factors, we

applied propensity score analysis with inverse probability weighting (IPW). Propensity scores reflect the probability that a patient will receive therapy based on observed covariates. By assigning propensity score weights to each patient and incorporating these weights into model construction, we can reduce treatment bias inherent in retrospective, non-randomized regression analyses.

The propensity score for each patient was estimated with a logistic regression model in which the treatment assignment was regressed on the clinicopathological variables including- sex, age, American Society of Anesthesiologists (ASA) classification, pT-stage and pN-stage.

Survival curves for the two groups were created with IPW-adjusted Kaplan-Meier plots, and 95% confidence intervals (CIs) were calculated with Cox proportional hazard models. Missing data were imputed using the single imputation method.

Additionally, we applied risk stratified analysis of treatment effect as an alternative to subgroup analyses [16-19]. Risk scores for developing recurrence were calculated for each patient provided their individual baseline characteristics of the variables included in the model. Each included variable represents a certain risk for the outcome. Depending on the value of these variables, risk scores are calculated per patient. Therefore, all patients have a risk score for the outcome regardless of adjuvant treatment. The variables include only those that were significantly associated with RFS from the Cox proportional hazards model and if they were clinically relevant to the outcome. The weight of the included variables on the risk scores are derived from the hazard ratios in the cox proportional hazards model. All patients were then subsequently ranked from lowest risk-score to highest and then split in different risk-groups, ranging from low risk to high risk. Survival was stratified for adjuvant treatment to analyze treatment effect. Treatment effects on both the relative scale (hazard ratio) and the absolute scale (absolute survival difference between treated and untreated patients) were estimated within each risk group and visualized by Kaplan-Meier cumulative recurrence and survival curves. The prediction model was visualized as a nomogram to enable use on plain paper and implementation as a calculation tool.

RESULTS

Data of 575 patients diagnosed with stage III colon cancer were included in this study. The median age at surgery was 68 years (interquartile range, IQR 61-76) and 52.3% of patients were male. In total, 353 patients (61.4%) had received adjuvant chemotherapy. Patients' demographics, pathological characteristics and treatment details are shown in Table 1.

Patients who did not receive adjuvant chemotherapy were older and had a higher ASA classification (both $p < 0.001$) (Table 1). In contrast, both T- and N stage were equally distributed in the two groups. Most patients had received capecitabine and oxaliplatin (CAPOX) (N=252, 71.3%). A small fraction received oxaliplatin and 5-FU (FOLFOX) (N=62, 17.6%), capecitabine monotherapy (N=28, 7.9%) or 5-FU monotherapy (n=2, 0.6%). Of 9 patients (0.3%) treatment details on which chemotherapy regimen was administered were missing. Most patients received at least half of the planned rounds of chemotherapy (n=289, 82.2%).

Table 1. Demographic variables

	All patients		**Chemotherapy**		**No chemotherapy**		**p-value**
	N=575	**%**	**N=353 (61.4%)**	**%**	**N=222 (38.6%)**	**%**	
Age							<0.001
<70	334	58.1	277	78.5	57	25.7	
≥70	241	41.9	76	22.5	165	74.3	
Median, IQR	68 (61-76)		65 (59-69)		76.5 (70-82)		
Gender							0.08
Male	301	52.3	195	55.2	106	47.7	
Female	274	47.7	158	44.8	116	52.3	
ASA-score							<0.001
I-II	367	81.6	249	88.3	118	70.2	
III-IIII	83	18.4	33	11.7	50	29.8	
Missing	125		71		54		
pT-stage							0.29
1	22	3.8	14	4.0	8	3.6	
2	86	15	48	13.7	38	17.1	
3	383	67.0	244	69.7	139	62.6	
4	81	14.2	44	12.6	37	16.7	
Missing	3		3				
pN-stage							0.95
1	388	67.7	238	67.8	150	67.6	
2	185	32.3	76	32.2	72	32.4	
Missing	2						
Tumor location							0.95
Right	255	44.8	149	42.6	106	48.4	
Left	314	55.2	201	57.4	113	51.6	
Missing	6		3		3		
Tumor differentiation							0.83
Good	8	1.4	4	1.1	4	1.8	
Moderate	412	71.7	256	72.5	156	70.3	
Poor	94	16.3	55	15.6	39	17.6	
Unknown/Other	61	10.6	38	10.8	23	10.4	

In the group of patients who did not receive chemotherapy (n=222), the treating physician had not recommended chemotherapy to 87 patients (39.2%), most often because of a combination of the patient's age and physical condition (n=47/87, 54%). Fifty-six patients (24.8%) refused chemotherapy despite recommendation; in 11 cases (19.6%) for fear of side-effects or loss of quality of life. Other documented reasons refraining patients from receiving adjuvant chemotherapy were a shared decision (n=19, 8.6%) and death before the start of chemotherapy (n=17, 7.7%). For 43 patients (19.4%) argumentation was not documented (Table 2).

Predictors for administration of adjuvant chemotherapy

Propensity scores were estimated with a multivariable logistic regression model in which treatment assignment (adjuvant chemotherapy) was regressed on clinicopathological variables to evaluate differences in the baseline characteristics between the two treatment groups. Age (linear) was the only significant predictor for receiving adjuvant chemotherapy (OR 0.12, 95% CI 0.08 – 0.18 $p < 0.001$) (Supplementary figure 1).

Table 2. Reasons for guideline non-adherence

	N	%
Doctors' recommendation	**87**	**39.2**
Condition/age	47	54.0
Treatment benefit not considered significant enough to recommend chemotherapy	8	9.2
Multidisciplinary recommendation	26	29.9
MSI	1	1.1
No argumentation	5	2.3
Patient rejection	**56**	**24.8**
Physical/mental condition	10	17.9
Fear of side effects/loss of quality of life	11	19.6
No argumentation	35	62.5
Shared decision	**19**	**8.6**
Age/performance status	15	78.9
No argumentation	4	21.1
Other	**17**	**7.7**
Deceased before starting chemo	17	100
No documentation	**43**	**19.4**

Effect of guideline adherence on survival and recurrence

Patients who died within 3 months of surgery were excluded from further analyses on survival as most of these patients (81%) were not able to start chemotherapy due to early death (n=27) resulting in 548 patients for the survival analyses.

The median follow-up for all patients was 47 months (IQR 27-62). The group of patients who were treated with surgery alone had a median follow-up of 32 months (IQR 17 – 54) compared to 52 months (IQR 38 - 64) for the group who received adjuvant chemotherapy (p <0.001). The median RFS for the surgery alone group was 70 months (95% CI 34 – 107 months), whereas the median RFS was not reached for the group who received chemotherapy. The group of patients who were treated with surgery alone had a median OS of 56 months (95% CI 46 - 66 months) and the median OS for the chemotherapy group was not reached. Crude

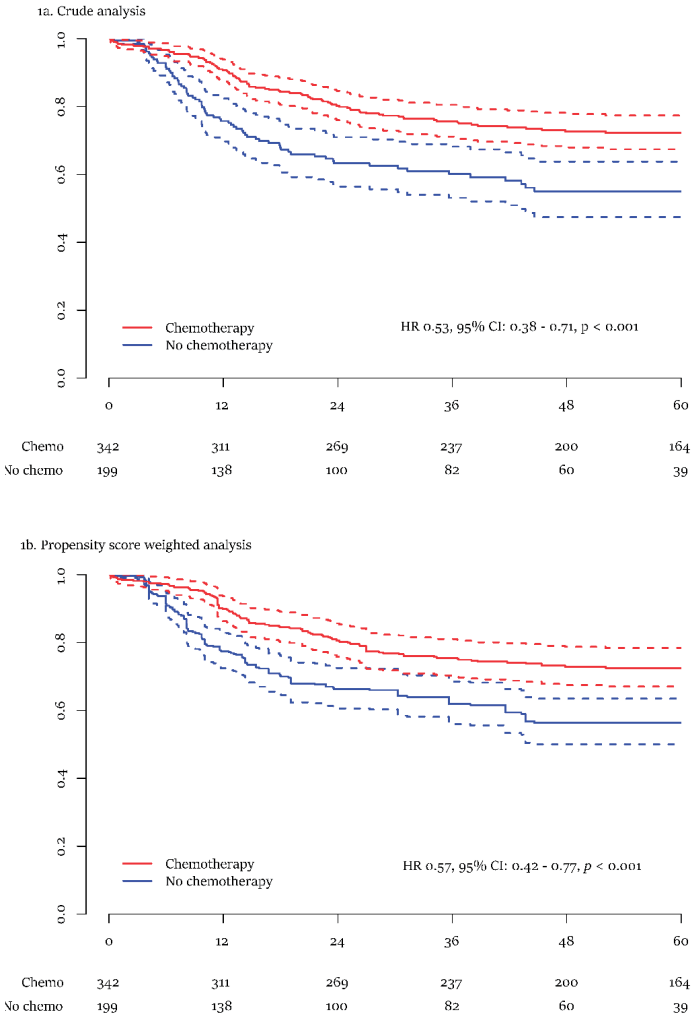
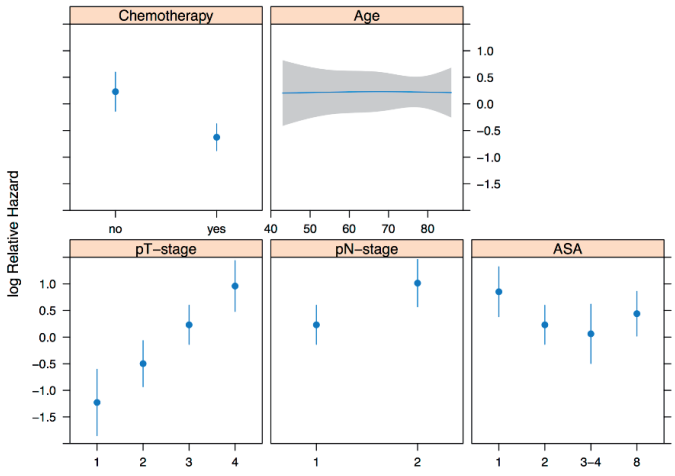


Figure 1. Treatment effect of adjuvant chemotherapy in stage III colon cancer patients. 1a. Crude analysis on RFS. 1b. Propensity score weighted analysis on RFS

Kaplan–Meier survival curves showed an impaired RFS (HR 0.53, 95% confidence interval (CI): 0.38–0.71), p < 0.001) for patients who did not receive adjuvant chemotherapy (fig 1A). After IPW was applied, RFS was still significantly worse for patients who were treated with surgery alone as compared with the chemotherapy-treated group (HR 0.57 95% CI 0.42-0.77 p < 0.001) (fig 1B).

Cox proportional hazards analyses did not reveal a significant association between reasons for non-adherence and patient survival when corrected for other variables (Supplementary table 1), nor did it reveal significant survival differences between the different chemotherapy regimens and survival (data not shown). In a Cox proportional hazard regression model, treatment with adjuvant chemotherapy was associated with longer RFS (HR 0.42, 95%CI 0.29 – 0.62, $p < 0.001$). High T-stage (HR 2.07, 95% CI 1.58 – 2.72, p < 0.001), N-stage (HR 2.19, 95% CI 1.60 – 2.99, $p < 0.001$) and ASA-score (HR 0.54, 95% CI 0.35 – 0.80 $p = 0.013$) were associated with significantly worse RFS (Figure 2).



	Multivariable		
	Hazard Ratio	**95% Confidence Interval**	***p*-value**
Age (years)	1.00	0.77 - 1.29	0.996
ASA-score	0.53	0.35 - 0.80	0.015
pT-stage	2.07	1.58 - 2.71	<0.001
pN-stage	2.19	1.60 - 2.99	<0.001
Adjuvant chemotherapy	0.42	0.29 - 0.62	<0.001

Figure 2. Cox proportional Hazards model on recurrence free survival

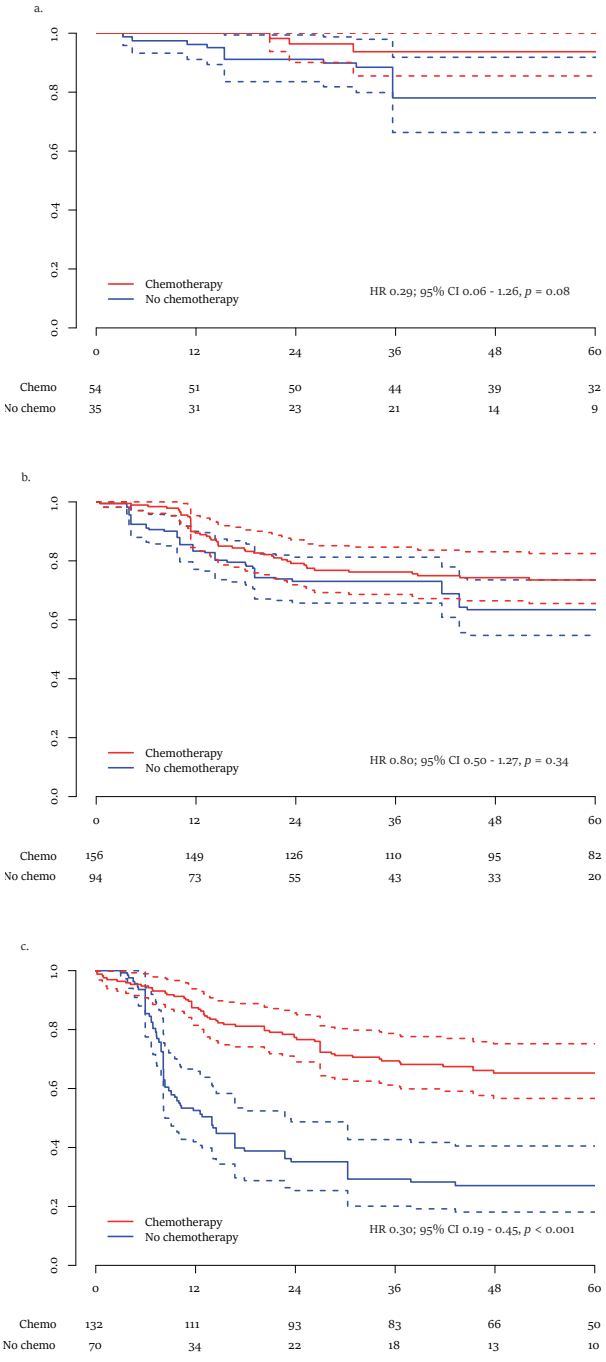


Figure 3. Kaplan Meier estimates of RFS among risk groups for patients with surgery plus adjuvant chemotherapy or surgery alone. **3a.** Risk group 1. **3b.** Risk group 2. **3c.** Risk group 3.

Risk-stratified analysis

As shown in the Cox proportional hazards model for RFS, T-stage, N-stage and ASA-score were associated with RFS. Only T-stage and N-stage are considered oncological relevant regarding the risk of recurrence and are therefore included in the risk-stratified analysis. The risk-stratified analysis of the effect of treatment with chemotherapy on RFS revealed clear differences in prognosis and treatment effect per group (Figure 3). Basic characteristics for the patients in the three developed risk groups are depicted per group in Table 3. The risk of developing recurrent disease without adjuvant chemotherapy is highest in the group with highest risk scores (group 3, HR 0.30, 95% CI 0.19-0.45, p<0.001) which includes most patients with T4 and N2 tumors. Is also shows that the absolute benefit of chemotherapy is much lower for the first two risk groups. However, based on the hazard ratios per group, there is no clear increase of treatment effect over the groups on RFS on a relative scale. We also performed OS analyses within these subgroups which demonstrates similar survival differences (Supplementary figure 2.)

Table 3. Characteristics of risk quartiles on recurrence free survival

	Risk group 1 N=90	**Risk group 2 N=250**	**Risk group 3 N=202**
Chemotherapy N (%)	55 (61.1)	156 (62.4)	132 (65.6)
Age Median (IQR)	66.7 (60-74)	67.4 (61-75)	68.6 (63-76)
ASA			
1	21 (23.3)	45 (18)	29 (14.4)
2	40 (44.4)	109 (43.6)	104 (51.5)
3-4	11 (12.2)	34 (13.6)	31 (15.3)
Unknown	18 (20)	62 (24.8)	38 (18.8)
Gender			
Male	51 (56.7)	137 (54.8)	100 (49.5)
Female	39 (43.3)	113 (45.2)	102 (50.5)
pT			
1	21 (23.3)	-	-
2	(69 (76.7)	12 (4.8)	-
3	-	238 (95.2)	128 (63.4)
4	-	-	74 (36.6)
pN			
1	89 (98.9)	238 (95.2)	40 (19.8)
2	1 (1.1)	12 (4.8)	162 (80.2)

Prognostic prediction nomogram

A prognostic nomogram was built using the same variables as in the risk stratified analyses to estimate the individual RFS for treatment with and without chemotherapy. Only T-stage and N-stage were included in the nomogram based on the prediction model for RFS (fig 4). By summing the scores associated with each variable and projecting total scores to the bottom scale, the probabilities can be estimated for RFS for surgery with or without adjuvant chemotherapy. When, after joint decision making between the treating physician and patient, it has been decided whether to start with adjuvant chemotherapy, this nomogram visualizes the individual RFS difference.

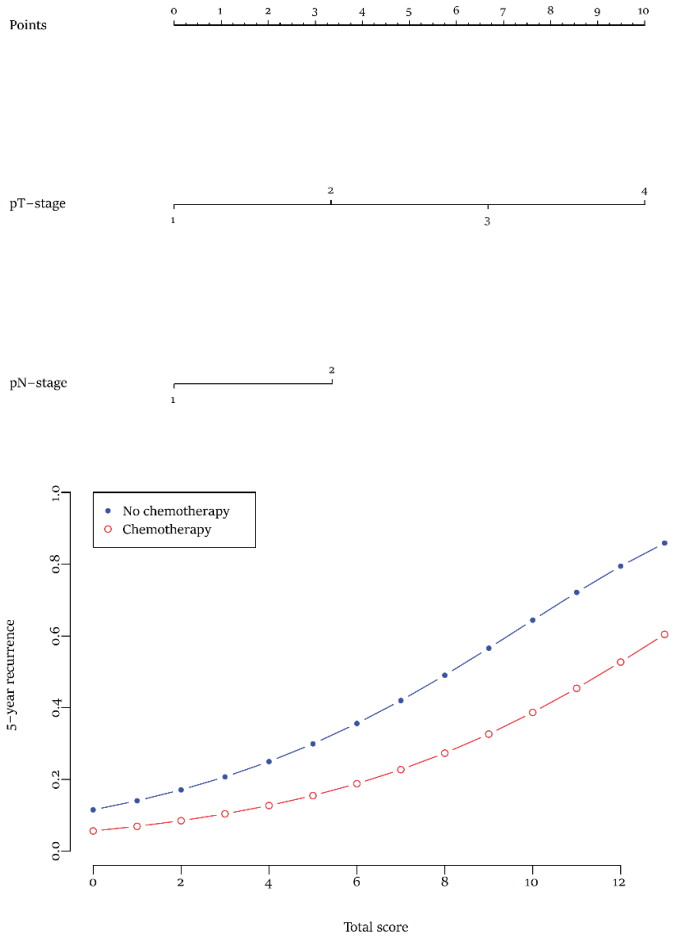


Figure 4. Nomogram of individualized treatment benefit from adjuvant chemotherapy on RFS.

Although relevant for daily practice, the above described nomogram analysis is not without a potential bias as the untreated group contains patients for which the reason to withhold adjuvant therapy was not reported. To avoid this possible bias, we made use of the subgroup of patients who refused adjuvant chemotherapy despite being eligible for treatment (N=56). We performed case-matching of these 56 patients based on T-stage and N-stage with 56 patients who did receive adjuvant chemotherapy. Thereby, a group of patients (N=112) was created who were all truly eligible for adjuvant chemotherapy but of which only 50% received chemo-

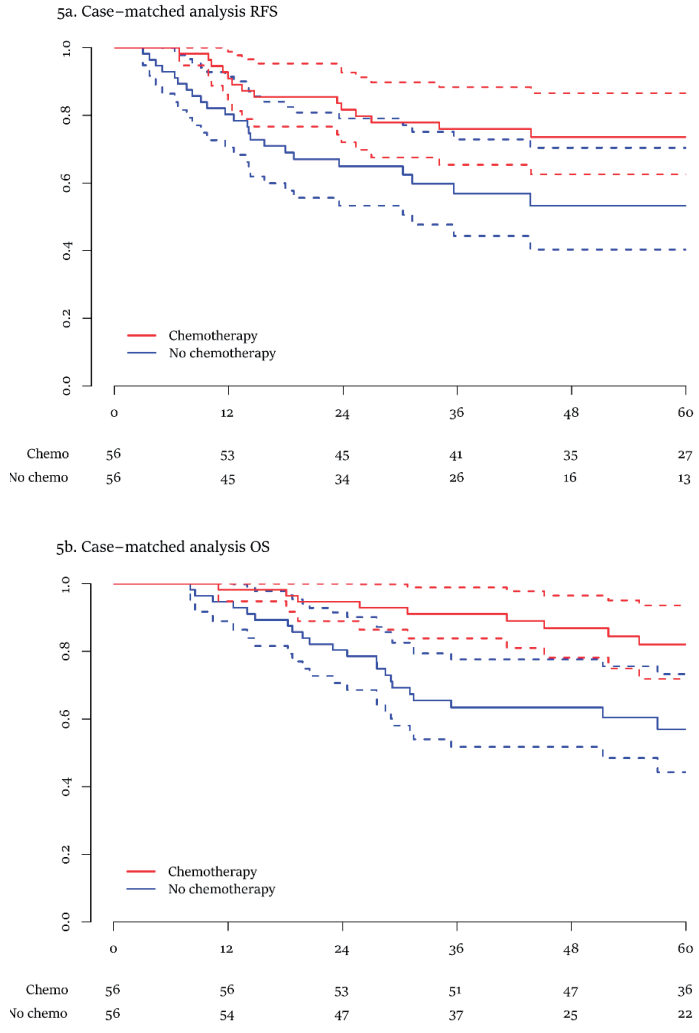


Figure 5. Subgroup survival analyses on RFS **(5a)** and OS **(5b)** stratified for adjuvant chemotherapy in eligible patients who refused chemotherapy, case-matched with patients who received chemotherapy.

therapy allowing for the analysis of treatment benefit. We calculated the average nomoscores of all these patients individually and composed the mean nomoscore (mean=7). Based on the nomogram this is expected to correspond to a 20% increase in RFS in the adjuvant-treated group. Intriguingly, the Kaplan Meier curve on RFS for this subgroup of patients show the predicted survival benefit from chemotherapy as can be derived from the nomogram (fig 5a). Kaplan Meier curves for OS within this group is also depicted in Figure 5b.

DISCUSSION

Guidelines recommend adjuvant chemotherapy for stage III colon cancer patients, given that they are fit to undergo chemotherapy. Our study shows that nearly 40% of the studied population with stage III colon cancer did not receive adjuvant chemotherapy following curative surgery. By carefully reviewing all patients' medical records, we found several reasons for refraining from adjuvant chemotherapy, however detailed reasons were found not to be documented in almost 40% of the patients who did not receive chemotherapy. High age was associated with reduced odds of receiving chemotherapy.

Not receiving adjuvant chemotherapy was significantly associated with worse RFS in analyses after applying inverse probability-weighting and correcting for confounding factors. Furthermore, risk-stratified analysis of the effect of chemotherapy on RFS revealed clear differences in prognosis and treatment effect between the risk groups. The absolute survival differences were larger for the high-risk patients suggesting a larger treatment benefit from adjuvant chemotherapy on RFS. Finally, we created a prognostic nomogram to estimate RFS difference for treatment with and without chemotherapy on an individual patient level.

Previous studies also showed that patients with stage III colon cancer patients may not receive adjuvant chemotherapy [22-26]. Unfortunately, we could also not find detailed information on the reasons for refraining from adjuvant chemotherapy in a significant number of patients (87/222, 39%). Thus, it remains unclear whether more patients could have had benefit from adjuvant chemotherapy or whether this subset did not receive chemotherapy as they were not fit enough or because of other counter-indications. Our results show a shorter median OS compared with the median RFS which indicates that a proportion of patients died before they could have developed a recurrence. This supports the importance of patient selection for adjuvant treatment because in these patients, adjuvant treatment would likely be more harmful with respect to quality of life rather than effective on survival because they die of other non-cancer related causes. Future research is mandated to investigate which treatment and decision strategy will optimally benefit patients with stage III colon cancer. A more detailed documentation may help to give further insight in the relevance and feasibility to adhere to the guidelines. Cancer

specialists in the fields of pathology, radiation, and surgery provide synoptic reporting in the form of pathology reports, radiation summaries, and operative reports. This type of documentation is essential for medical oncologists as well because treatment often takes many months and visits, and may be complicated by discontinuity caused by mental as well as physical limitations. There have been initiatives to improve documentation. For instance, a group of ASCO members developed the "Chemotherapy Treatment Summary: A Two-Step Process" which helps to clearly document and summarize the individual treatment [27]. More efforts for implementing better and more structured documentation should be undertaken.

In the present study, comorbidity and high age were reasons to withheld adjuvant chemotherapy in almost one third of the cases, as documented in patients' charts. Older, more fragile patients can suffer more from chemotherapy toxicity than younger, fitter patients which could be a valid reason for withholding chemotherapy. Interestingly, ASA-score – a grading system for preoperative health of the surgical patients – was not independently associated with receiving chemotherapy. However, ASA-score is designed as a measure for patients' health for undergoing surgery, not for administering chemotherapy. Furthermore, T- and N-stage were not associated with the odds of receiving chemotherapy while T4-stage and N2-stage are associated with higher rates of recurrence and shorter survival [28]. These results further underline the importance of proper documentation to analyze to whether these factors are considered in clinical practice, and how this affects patient outcome. Multiple studies, including ours, have shown that adjuvant chemotherapy can have a significant effect on survival. On the other hand, it also influences quality of life. For future studies, it would be interesting to perform a discrete choice experiment to analyze which factors influence patients' choices, and trade-offs in survival that patients are willing to make in their choice between surgery and surgery plus adjuvant chemotherapy.

We performed a risk-stratified analysis of treatment effect as an alternative to subgroup analyses [17-19, 29, 30]. The most used method of examining whether treatment effects vary in a population is to serially divide patients into subgroups based on potentially relevant pre-treatment characteristics. However, this leads to numerous subgroup analyses, which are vulnerable to false-positive results due to multiple comparisons. Kent et al concluded that in risk stratified analysis, the treatment benefit is often higher for the higher-risk patients [16]. Contrarily, treatment with estimated overall benefit may even be harmful to low-risk patients, considering the positive probability of treatment-related harm. This is concurrent with our results where patients from the highest risk groups seem to have a larger treatment benefit. Our study shows that there are clear differences in survival and treatment effect per risk group. The risk of developing recurrent disease without adjuvant chemotherapy is highest in the group with the highest risk scores. For the first two risk groups on RFS, there seems to be no significant survival difference for patients treated with or without adjuvant chemotherapy. These groups comprise patients with low T- and N-stage tumors.

The current study provides a nomogram that may help both physicians as well as patients in shared decision-making and personalized treatment. The nomogram visualizes that withholding chemotherapy in patients with low T- and N-stage may be justified at the expense of a small impact on the RFS, for instance in patients with a poor physical or mental health. On the other hand, when a patient with a high T- and/or N-stage tumor has doubts on starting chemotherapy, it can also help in visualizing the large survival gain which can be won by chemotherapy.

Our study shows a 3-year rate of RFS of 76.3% for the chemotherapy treated patients and 66.8% for the patients who were treated with surgery alone, which is line with previous results from other large randomized studies [7, 31, 32]. Furthermore, other 'real-world' data from the Surveillance, Epidemiology, and End Results registry linked to Medicare claims (SEER-Medicare), the New York State Cancer Registry linked to Medicaid and Medicare claims (NYSCR), the National Comprehensive Cancer Network (NCCN) Outcomes Database, and the Cancer Care Outcomes Research & Surveillance Consortium (CanCORS), demonstrate that adjuvant chemotherapy is beneficial across all age groups [33, 34]. However, McCleary 2013 et al. suggests that for patients age > 70 years, oxaliplatin may provide a DFS benefit only for a subset of older adults but could not establish which subsets of older adults experiences this benefit [35].

Several limitations of the study need to be addressed. First, treatment assignment had an inherent selection bias, as shown from the baseline differences between the two groups. Decisions to administer chemotherapy seem to have been based mostly on age. Although propensity-matching using IPW estimators may be the best statistical strategy to adjust for covariates in a retrospective cohort, such methodology is not a substitute for a prospective randomized controlled trial. Ideally, a nomogram like the one presented in this study would be built on data prospectively collected from a randomized controlled trial where two groups are compared: patients treated with surgery followed by a combination of chemotherapy (i.e. CAPOX or FOLFOX) versus patients treated with surgery alone. However, due to the widely proved overall survival benefit of adjuvant chemotherapy in this patient selection, such a study is highly unethical and will therefore most likely never be performed. Therefore, retrospective data is second best to analyze individual treatment benefit. We analyzed the effect of adjuvant chemotherapy in eligible patients based on tumor stage. However, 'eligibility' also comprises patient's physical health and co-medication. Even though we adjusted for ASA-score, as mentioned before, this is not a grading system designed for administering chemotherapy and does not comprise all comorbidities which can affect eligibility for chemotherapy. Inherently, we did not control for some comorbidities which can influence treatment decisions regarding administering chemotherapy and thereby patient outcomes. Co-medication and performance status are two important factors which influence eligibility.

Second, we did not include biological tumor characteristics in the survival analyses. It it has been observed that MSI predicts lack of response to conventional chemotherapy [36-38]. Furthermore, recent studies suggest that biological subtypes in colon cancer (consensus molecular subtypes) have a different response to chemotherapy [39]. Future studies should aim to incorporate both clinical and tumor characteristics in nomograms. Another clinically relevant limitation is that we have no information on which physician – surgeon or medical oncologist – discussed with the patient whether adjuvant chemotherapy should be administered. While non-receipt of adjuvant therapy may be entirely appropriate given the circumstances of individual patients, one could argue that having a discussion or consultation regarding the adjuvant therapy with a physician who specializes in adjuvant therapies represents the ideal management scenario for patients with potentially curable disease and in whom adjuvant therapy has been shown to be beneficial. Our study shows a considerable number of patients who refrain from chemotherapy themselves, for instance due to fear of side-effects. The analysis on this subgroup of patients clearly indicates a significant benefit of chemotherapy for this group of patients on RFS and OS. A medical oncologist can explain that new guidelines advise on treatment with three months of adjuvant CAPOX instead of six months, which results in significantly less severe side-effects [7]. In addition, an oncologist can advise on monotherapy with fluoropyrimidine if side-effects from oxaliplatin are expected to be worse in older, fragile patients. Though there is no defined "ideal" benchmark for referral or consultation rates, both referral to and consultation with a medical oncologist have been identified as measures of quality care for patients with resected (or resectable) disease and large variations exist in referral patterns by patient characteristics for resected colon cancer patients [40-42].

In conclusion, this study shows considerable variation in adherence to the national guidelines with respect to treatment with adjuvant chemotherapy in stage III colon cancer. Age seems to be a large contributing factor for not administering adjuvant chemotherapy but generally the reasons for such are poorly documented. While non-receipt of adjuvant therapy may be entirely appropriate given the circumstances of individual patients, documentation of these considerations is essential for research and advancement of clinical practice. Especially because not administering adjuvant chemotherapy can lead to a significant recurrence free survival difference, most present in patients with high T-stage and N-stage tumors. The nomogram in this study may be helpful for the introductory conversation on adjuvant chemotherapy. Furthermore, future efforts should be aimed to improve consultation and documentation by implementing methods such as synoptic reporting on reasons to either administer adjuvant chemotherapy or to refrain from it. Lastly, it would be very interesting for future studies to analyze whether the recent change in guidelines on adjuvant chemotherapy duration from 6 to 3 months influences guideline adherence, for instance on the number of patients refusing chemotherapy for fear of side-effects.

REFERENCES

1. Nederlandse Kankerregistratie (NKR), IKNL.
2. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, et al. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. *Eur J Cancer* 2013; 49(6):1374-403.
3. Labianca R, Nordlinger B, Beretta GD, et al. Early colon cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2013; 24 Suppl 6:vi64-72.
4. André T, Boni C, Navarro M, et al. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. *J Clin Oncol* 2009; 27(19):3109-3116.
5. Haller DG, Tabernero J, Maroun J, et al. Capecitabine plus oxaliplatin compared with fluorouracil and folinic acid as adjuvant therapy for stage III colon cancer. *J Clin Oncol* 2011; 29(11):1465-1471.
6. Moertel CG, Fleming TR, Macdonald JS, et al. Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. *N Engl J Med* 1990; 322(6):352-8.
7. Grothey A, Sobrero AF, Shields AF, et al. Duration of Adjuvant Chemotherapy for Stage III Colon Cancer. *N Engl J Med* 2018; 378(13):1177-1188.
8. Ananda S, Field KM, Kosmider S, et al. Patient age and comorbidity are major determinants of adjuvant chemotherapy use for stage III colon cancer in routine clinical practice. *J Clin Oncol* 2008; 26(27):4516-7; author reply 4517-8.
9. Lemmens VE, van Halteren AH, Janssen-Heijnen ML, et al. Adjuvant treatment for elderly patients with stage III colon cancer in the southern Netherlands is affected by socioeconomic status, gender, and comorbidity. *Ann Oncol* 2005; 16(5):767-72.
10. Winget M, Hossain S, Yasui Y, et al. Characteristics of patients with stage III colon adenocarcinoma who fail to receive guideline-recommended treatment. *Cancer* 2010; 116(20):4849-56.
11. Quipourt V, Jooste V, Cottet V, et al. Comorbidities alone do not explain the undertreatment of colorectal cancer in older adults: a French population-based study. *J Am Geriatr Soc* 2011; 59(4):694-8.
12. Jorgensen ML, Young JM, Solomon MJ. Adjuvant chemotherapy for colorectal cancer: age differences in factors influencing patients' treatment decisions. *Patient Prefer Adherence* 2013; 7:827-34.
13. Keating NL, Landrum MB, Klabunde CN, et al. Adjuvant chemotherapy for stage III colon cancer: do physicians agree about the importance of patient age and comorbidity? *J Clin Oncol* 2008; 26(15):2532-7.
14. Levine RA, Chawla B, Bergeron S, et al. Multidisciplinary management of colorectal cancer enhances access to multimodal therapy and compliance with National Comprehensive Cancer Network (NCCN) guidelines. *Int J Colorectal Dis* 2012; 27(11):1531-8.
15. Austin PC. The performance of different propensity score methods for estimating marginal hazard ratios. *Stat Med* 2013; 32(16):2837-49.
16. Kent DM, Nelson J, Dahabreh IJ, et al. Risk and treatment effect heterogeneity: re-analysis of individual participant data from 32 large clinical trials. *Int J Epidemiol* 2016; 45(6):2075-2088.
17. Kent DM, Paulus JK, van Klaveren D, et al. The Predictive Approaches to Treatment effect Heterogeneity (PATH) Statement. *Ann Intern Med* 2020; 172(1):35-45.
18. Kent DM, Steyerberg E, van Klaveren D. Personalized evidence based medicine: predictive approaches to heterogeneous treatment effects. *BMJ* 2018; 363:k4245.

19. Kent DM, van Klaveren D, Paulus JK, et al. The Predictive Approaches to Treatment effect Heterogeneity (PATH) Statement: Explanation and Elaboration. *Ann Intern Med* 2020; 172(1):W1-W25.
20. van Vugt JLA, Coebergh van den Braak RRJ, Lalmahomed ZS, et al. Impact of low skeletal muscle mass and density on short and long-term outcome after resection of stage I-III colorectal cancer. *Eur J Surg Oncol* 2018; 44(9):1354-1360.
21. Lalmahomed ZS, Coebergh van den Braak RRJ, Oomen MHA, et al. Multicenter fresh frozen tissue sampling in colorectal cancer: does the quality meet the standards for state of the art biomarker research? *Cell Tissue Bank* 2017; 18(3):425-431.
22. van Steenbergen LN, Rutten HJ, Creemers GJ, et al. Large age and hospital-dependent variation in administration of adjuvant chemotherapy for stage III colon cancer in southern Netherlands. *Ann Oncol* 2010; 21(6):1273-8.
23. Rayson D, Urquhart R, Cox M, et al. Adherence to clinical practice guidelines for adjuvant chemotherapy for colorectal cancer in a Canadian province: a population-based analysis. *J Oncol Pract* 2012; 8(4):253-9.
24. Renouf D, Kennecke H, Gill S. Trends in chemotherapy utilization for colorectal cancer. *Clin Colorectal Cancer* 2008; 7(6):386-9.
25. Cree M, Tonita J, Turner D, et al. Comparison of treatment received versus long-standing guidelines for stage III colon and stage II/III rectal cancer patients diagnosed in Alberta, Saskatchewan, and Manitoba in 2004. *Clin Colorectal Cancer* 2009; 8(3):141-5.
26. Keikes L, van Oijen MGH, Lemmens V, et al. Evaluation of Guideline Adherence in Colorectal Cancer Treatment in The Netherlands: A Survey Among Medical Oncologists by the Dutch Colorectal Cancer Group. *Clin Colorectal Cancer* 2018; 17(1):58-64.
27. Developing the medical oncology treatment plan and summary. *J Oncol Pract* 2006; 2(2):95-6.
28. Gunderson LL, Jessup JM, Sargent DJ, et al. Revised TN categorization for colon cancer based on national survival outcomes data. *J Clin Oncol* 2010; 28(2):264-71.
29. Kent DM, Paulus JK, van Klaveren D, et al. The Predictive Approaches to Treatment effect Heterogeneity (PATH) Statement. *Ann Intern Med* 2019.
30. Kent DM, van Klaveren D, Paulus JK, et al. The Predictive Approaches to Treatment effect Heterogeneity (PATH) Statement: Explanation and Elaboration. *Ann Intern Med* 2019.
31. Andre T, Boni C, Navarro M, et al. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. *J Clin Oncol* 2009; 27(19):3109-16.
32. Quasar Collaborative G, Gray R, Barnwell J, et al. Adjuvant chemotherapy versus observation in patients with colorectal cancer: a randomised study. *Lancet* 2007; 370(9604):2020-9.
33. Sanoff HK, Carpenter WR, Sturmer T, et al. Effect of adjuvant chemotherapy on survival of patients with stage III colon cancer diagnosed after age 75 years. *J Clin Oncol* 2012; 30(21):2624-34.
34. Sanoff HK, Carpenter WR, Martin CF, et al. Comparative effectiveness of oxaliplatin vs non-oxaliplatin-containing adjuvant chemotherapy for stage III colon cancer. *J Natl Cancer Inst* 2012; 104(3):211-227.
35. McCleary NJ, Meyerhardt JA, Green E, et al. Impact of age on the efficacy of newer adjuvant therapies in patients with stage II/III colon cancer: findings from the ACCENT database. *J Clin Oncol* 2013; 31(20):2600-6.

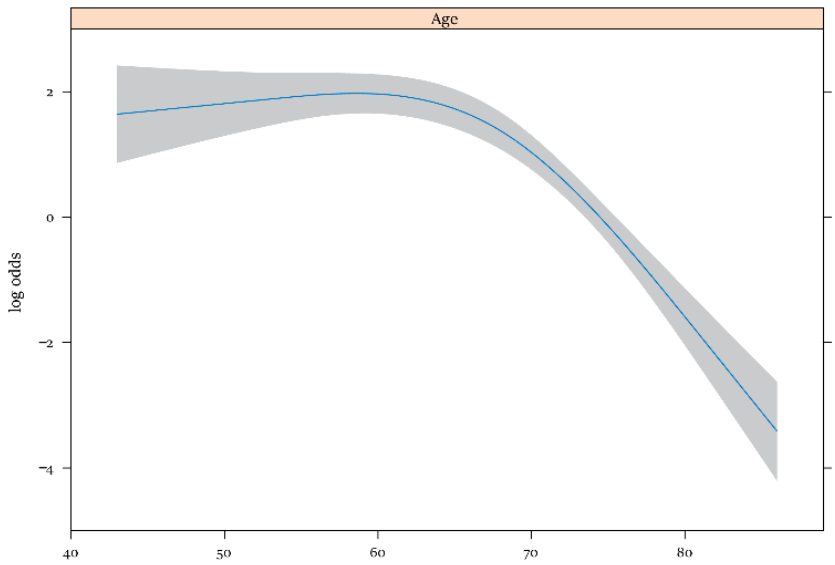
36. Des Guetz G, Lecaille C, Mariani P, et al. Prognostic impact of microsatellite instability in colorectal cancer patients treated with adjuvant FOLFOX. *Anticancer Res* 2010; 30(10):4297-301.
37. Des Guetz G, Schischmanoff O, Nicolas P, et al. Does microsatellite instability predict the efficacy of adjuvant chemotherapy in colorectal cancer? A systematic review with meta-analysis. *Eur J Cancer* 2009; 45(10):1890-6.
38. Sargent DJ, Marsoni S, Monges G, et al. Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. *J Clin Oncol* 2010; 28(20):3219-26.
39. Guinney J, Dienstmann R, Wang X, et al. The consensus molecular subtypes of colorectal cancer. *Nat Med* 2015; 21(11):1350-6.
40. Ong S, Watters JM, Grunfeld E, et al. Predictors of referral for adjuvant therapy for colorectal cancer. *Can J Surg* 2005; 48(3):225-9.
41. Gagliardi AR, Simunovic M, Langer B, et al. Development of quality indicators for colorectal cancer surgery, using a 3-step modified Delphi approach. *Can J Surg* 2005; 48(6):441-52.
42. McGory ML, Shekelle PG, Ko CY. Development of quality indicators for patients undergoing colorectal cancer surgery. *J Natl Cancer Inst* 2006; 98(22):1623-33.

Supplementary Data

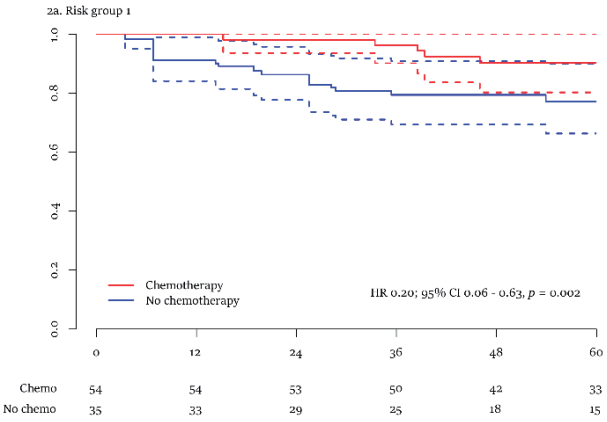
5

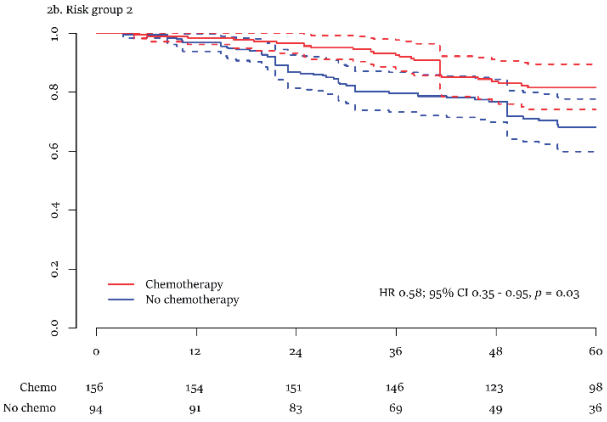
Supplementary table 1. Reasons for non-adherence and RFS

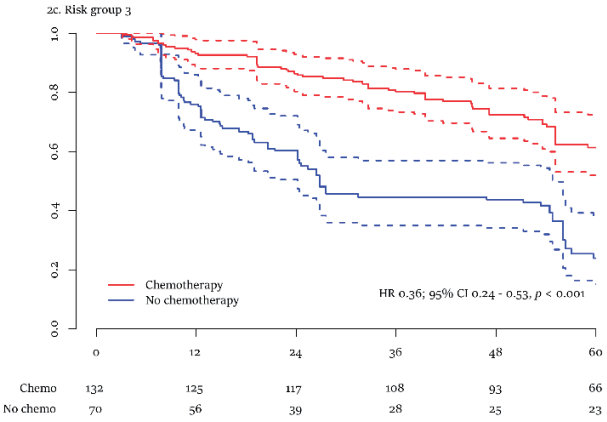
	Multivariable		
	Hazard Ratio	**95% CI**	**p-value**
Age	0.99	0.96 – 1.02	0.631
ASA-score (I+II = Ref)	0.69	0.35 - 1.36	0.281
Tumor location (Right = Ref)	0.50	0.27 – 0.94	0.032
pT			
1 (Ref)		-	0.162
2	1.83	0.22 – 15.18	0.576
3	2.08	0.27 – 15.84	0.479
4	4.26	0.52 – 35.25	0.179
pN-stage (N1 = Ref)	2.71	1.48 – 4.99	0.001
Tumor differentiation grade			
Good (Ref)		-	0.909
Moderate	3499.36	0.00 – 2.03[56]	0.895
Poor	4086.12	0.00 – 2.38[56]	0.893
Unknown	4759.07	0.00 – 2.77[56]	0.891
Patients' choice	.		0.537
Doctors' recommendation	0.84	0.43 – 1.67	0.625
Shared decision	0.71	1.95 – 1.84	0.476
No documentation	0.54	0.24 – 1.26	0.155



Supplementary figure 1. Propensity score model for the effect of age on administering adjuvant chemotherapy







Supplementary figure 2. Overall survival analyses stratified by adjuvant chemotherapy per RFS risk group. **2a.** Risk group 1. **2b.** Risk group 2. **2c.** Risk group 3.

Chapter 6

Low socioeconomic status is associated with worse outcomes after curative surgery for colorectal cancer: Results from a large, multicenter study

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ABSTRACT

Background

Socioeconomic status (SES) has been associated with early mortality in cancer patients. However, the association between SES and outcome in colorectal cancer patients is largely unknown. The aim of this study was to investigate whether SES is associated with short and long-term outcome in patients undergoing curative surgery for colorectal cancer.

Methods

Patients who underwent curative surgery in the region of Rotterdam for stage I-III colorectal cancer between January 2007 and July 2014 were included. Gross household income and survival status were obtained from a national registry provided by Statistics Netherlands (CBS). Patients were assigned percentiles according to the national income distribution. Logistic regression and Cox proportional hazard regression were performed to assess the association of SES with 30-day postoperative complications, overall survival, and cancer specific survival, adjusted for known prognosticators.

Results

For 965 of the 975 eligible patients (99%), gross household income could be retrieved. Patients with a lower SES more often had diabetes, more often underwent an open surgical procedure, and had more comorbidities. In addition, patients with a lower SES were less likely to receive (neo)adjuvant treatment. Lower SES was independently associated with an increased risk of postoperative complications (Odds ratio per percent increase 0.99, 95%CI 0.99–0.998, p=0.004) and lower cancer-specific mortality (Hazard ratio per percent increase 0.99, 95%CI 0.98-0.99, p=0.009).

Conclusion

This study shows that lower SES is associated with increased risk of postoperative complications, and poor cancer-specific survival in patients undergoing surgery for stage I-III colorectal cancer after correcting for known prognosticators.

INTRODUCTION

With an incidence of 464,800 patients in 2012, colorectal cancer (CRC) is the second most common cancer in Europe and constitutes a large burden, both in economic and in medical terms [1, 2]. In a recent European article, average costs for treating a single patient with colorectal cancer were estimated at 40.000 Euros [2]. Currently, the TNM classification is the most important determinant for treatment decisions and outcome. Resection still remains the only cure, and the 5-year survival rate for patients with stage I–III varies between 53 and 92% [1]. Still, there are individual differences in clinical outcome within a single tumour stage.[3] Next to these tumour characteristics, several patient factors such as obesity, diabetes mellitus, smoking and nutritional status have been investigated and associated with survival, yet much of the disparity in prognosis remains unexplained [4-7].

A possible explanation for differences in survival is socio-economic status (SES). Not only does this influence the ability to pay medical bills and having financial resources to follow through with all hospital check-ups, other correlating factors such as obesity and diabetes may also have an influence on post-resection CRC survival [8-10]. In previous studies, SES has been investigated as a possible prognostic factor for survival in cancer patients with contradicting results [11-23]. Some of this variability in outcomes may be explained by accessibility of healthcare [22, 23]. Some studies have been performed in countries where access to and the quality of health care is directly linked to income (e.g. United States) [22, 23], whereas others were conducted in countries with equal healthcare access (e.g. Great Britain and Scandinavia) [11-23]. The Dutch healthcare system is known for its equal healthcare access[24, 25], meaning that differences in outcome associated with SES can be attributed to patient and provider factors and their interaction, rather than disparities in healthcare [26]. In the current study we aimed to explore the association between SES, as assessed by household income, and outcomes following curative resection for stage I-III colorectal cancer in the Netherlands.

METHODS

Study population

Patients with stage I-III colorectal cancer who underwent curative surgery, enrolled in the MATCH-study between 2007 and 2014, were included in this study. The MATCH study is a prospective multicentre cohort study including patients to obtain fresh frozen CRC tissue samples with matched clinical data from 2007 until December 2017 in 6 hospitals in the region of Rotterdam.[27]

The rationale of the MATCH study was to identify subtypes of colorectal cancer, related prognostic markers and outcome of treatment. [28]. The study was approved by the Erasmus MC IRB (MEC-2007-088) and all patients provided written informed consent.

Study parameters

Socioeconomic status was defined as gross household income (GHI), the most commonly used and accepted surrogate marker for SES [26, 29-31]. GHI from the year prior to surgery was used for analyses, as the income in the year of surgery was possibly lower due to disease-related absence from work. Annual earnings were obtained from Statistics Netherlands, a governmental organisation enabling studies on social issues on the basis of reliable statistical information (Centraal Bureau voor de Statistiek; CBS), including all types of income of people sharing a household or place of residence combined (i.e. salary, state pension, social compensation, and investment revenues). Patients were assigned percentiles and quartiles (Q1-Q4) according to the national income distribution (i.e. patients of a household with an annual salary corresponding to 0-25% of the GHI of the Dutch population were stratified to the first income quartile). Baseline characteristics and variables were retrieved from the prospective database of the MATCH study.

Dutch healthcare system

The current Dutch healthcare system was introduced on January 1st 2006. All Dutch citizens are legally required to have health care insurance offered by several private insurers. Basic insurance premiums have a legal maximum and allowances are available for the lower incomes. Basic insurances cover all medical costs concerning regular cancer care including all in hospital care, costs for medicines, cancer rehabilitation, emergency transfer to hospitals, dietary help and psychosocial assistance. Out-of-pocket expenses such as transportation represented 14.7 percent of health care spending in 2014 [32].

Outcome measures

The primary endpoints were overall survival (OS) and cancer specific survival (CSS) after surgery for colorectal cancer, calculated from the day of surgery to the day of death (from disease) or loss to follow-up, whichever came first. Date and cause of death were obtained from the national registry of Statistics Netherlands. Secondary outcome measures were 30-day postoperative complications. Severity of complications was scored according to the Clavien-Dindo classification and major 30-day postoperative complications were defined as Clavien-Dindo score ≥3a [33].

Statistical methods

Descriptive statistics and multivariable analyses were performed using SPSS Version 24.0 (SPSS, Inc., Chicago, IL, USA). Differences in baseline characteristics between the SES quartiles were tested with Pearson's Chi-square analysis or Mann-Whitney U-test as appropriate. Logistic regression analysis was used to calculate the odds ratio (OR) with 95% confidence intervals (95% CI) to evaluate the influence of SES, patient and tumour characteristics and operation techniques on 30-day postoperative complications. The outcomes OS and CSS were analysed with Cox proportional hazards regression. Unadjusted differences in the survival between income quartiles were assessed using the log-rank test. The predictors included in regression models were selected based on clinical relevance based on previous literature. Two-sided *p*-values <0.05 were considered statistically significant.

RESULTS

A total of 975 patients met the inclusion criteria. For 10 patients, gross household income (GHI) could not be retrieved, leaving a final sample size of 965 (99%) patients. Patients with low SES were more often female and older (both $p < 0.001$) (Table 1).

Diabetes mellitus and higher American Society of Anesthesiologists (ASA) classification were more common among patients with low SES (both $p = 0.001$). The Charlson Comorbidity Index was significantly higher for patients in the lowest quartile ($p < 0.001$). The median length of stay (LOS) was higher for patients in the lowest quartile ($p < 0.001$). Treatment strategies also differed between the quartiles; patients with low SES more often underwent open surgery ($p = 0.003$) and less often received neoadjuvant and adjuvant treatment ($p = 0.037$ and $p = 0.001$, respectively).

SES and postoperative complications

A total of 443 patients (45.9%) suffered at least one postoperative complication. The overall complication rate gradually decreased from 53.3% in Q1 to 36.0% in Q4 ($p < 0.001$). Major complications (i.e. Clavien-Dindo score ≥3) occurred in 170 patients (17.6%), for which a similar gradual decrease was observed (21.3% in Q1 to 12.4% in Q4; $p < 0.001$). No significant differences in readmission and reoperation rates were observed between the quartiles (Figure 1).

Table 1. Baseline characteristics

	Q1	%	Q2	%	Q3	%	Q4	%	p-value
	(n=244)		(n=348)		(n=187)		(n=186)		
Demographics									
Sex female	146	(59.8)	153	(44)	75	(40.1)	63	(33.9)	<0.001
Age (median, IQR)	76	(69-81)	72	(66-78)	66	(60-72)	59	(55-65)	<0.001
BMI (median, IQR)	25.9	(23.3-29.4)	25.9	(23.5-28.5)	25.8	(23.0-28.3)	25.4	(23.4-28.7)	0.795
CEA preoperative (median, IQR)	3.3	(2.0-8.0)	3.5	(2.0-8.0)	3.3	(1.85-6.9)	2.9	(1.9-5.75)	0.160
Comorbid conditions									
Charlson Comorbidity Index (median, IQR)	1	(0.0-2.0)	1	(0-2.0)	0	(0-1.0)	0	(0-0.1)	<0.001
Diabetes mellitus	62	(25.5)	72	(20.7)	28	(15)	20	(10.8)	0.001
COPD	27	(11.1)	29	(8.3)	14	(7.5)	12	(6.5)	0.344
ASA									
I-II	189	(78.1)	274	(79)	159	(86.4)	168	(90.8)	0.001
III+	53	(21.9)	73	(21)	25	(13.6)	17	(9.2)	
Missing	2		1		3		1		
Surgical technique									
Open	121	(49.8)	154	(44.3)	66	(35.9)	77	(41.6)	0.003
Laparoscopic	102	(42)	168	(48.3)	105	(57.1)	80	(43.2)	
Conversion	20	(8.2)	26	(7.5)	13	(7.1)	28	(15.1)	
Missing	1		0		3		1		
Stoma	69	(38.3)	106	(30.5)	61	(33)	55	(29.7)	0.768
Missing	0		1		2		1		
Length of stay (LOS)	9	(6-14)	7.5	(6-13)	7	(5-10)	7	(5-12)	<0.001
Blood loss (L)	0.159	(0.05-0.40)	1	(0.03-0.40)	0.11	(0.04-0.25)	0.1	(0.02-0.35)	0.065

Table 1. Baseline characteristics (continued)

	Q1	%	Q2	%	Q3	%	Q4	%	p-value
	(n=244)		(n=348)		(n=187)		(n=186)		
Operation duration (hours)	2.53	(1.83-3.28)	2.47	(1.83-3.25)	2.5	(2.0-3.2)	2.67	(1.87-3.52)	0.356
Neoadjuvant therapy	57	(23.4)	84	(24.2)	63	(33.7)	57	(30.6)	0.037
Adjuvant chemotherapy	28	(11.6)	60	(17.3)	40	(21.4)	50	(26.9)	0.001
Missing	3		2		0		0		
Tumour characteristics									
Tumour stage									
I	82	(33.6)	107	(30.7)	52	(27.8)	64	(34.4)	0.278
II	82	(33.6)	139	(39.9)	69	(36.9)	56	(30.1)	
III	80	(32.8)	102	(29.3)	66	(35.3)	66	(35.5)	
Tumour grade differentiation									
Good	59	(25.5)	89	(26.6)	54	(29.7)	48	(26.4)	0.534
Moderate	144	(62.3)	214	(64.1)	105	(57.7)	120	(65.9)	
Poor	28	(12.1)	31	(9.3)	23	(12.6)	14	(7.7)	
Missing	13		14		5		4		
Angioinvasion									
No	65	(28.8)	94	(28.8)	60	33.9)	57	(32.8)	0.749
Yes	25	(11.1)	29	(8.9)	15	8.5)	13	(7.5)	
Not reported	136	(60.2)	203	(62.3)	102	57.6)	104	(59.8)	
Missing	18		22		10		12		
Location of tumour - rectum	64	(26.2)	99	(28.4)	66	(35.3)	67	(36)	0.059

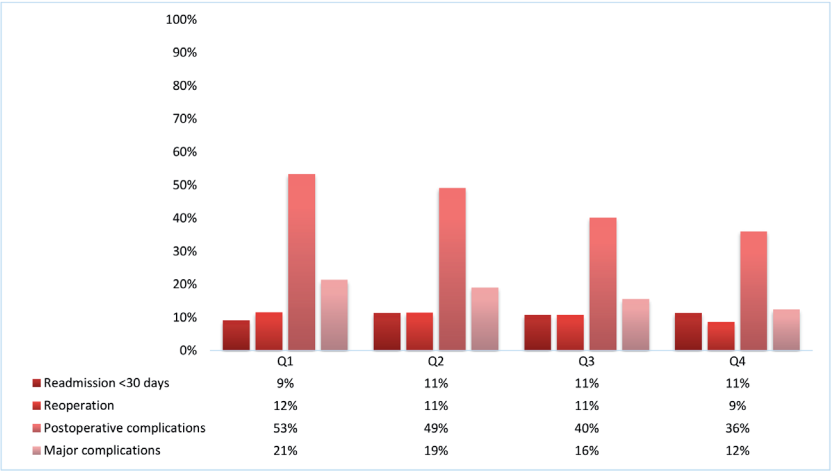

Figure 1. Postoperative complications per quartile

Table 2. Univariable and multivariable analyses on overall postoperative complications

	Univariable			Multivariable		
	Odds Ratio	95% Confidence Interval	p-value	Odds Ratio	95% Confidence Interval	p-value
Gender – Male = reference	0.84	0.65 – 1.08	0.174	0.79	0.60 – 1.00	0.086
Age (years)	1.02	1.01 – 1.04	<0.001	1.02	1.00 – 1.03	0.044
Charlson comorbidity	1.09	1.00 – 1.19	0.051	1.03	0.94 – 1.00	0.497
Rectal carcinoma	1.56	1.19 – 2.05	0.001	1.81	1.00 – 2.00	<0.001
Surgical technique - Open = reference	0.56	0.44 – 0.73	<0.001	0.56	0.42 – 0.73	<0.001
Gross household income	0.99	0.99 – 0.99	<0.001	0.99	0.99 – 0.998	0.004

In univariable logistic regression, GHI was associated with overall postoperative complications (OR 0.99, 95%CI 0.99 – 0.99, $p < 0.001$), as was older age (OR 1.02, 95%CI 1.01 – 1.04, $p < 0.001$), rectal cancer (OR 1.56, 95%CI 1.19 – 2.05, $p = 0.001$), and open surgery (OR 0.56, 95% CI 0.44 – 0.73, $p < 0.001$). The association between GHI and overall postoperative complications remained significant in the multivariable model (OR 0.99, 95%CI 0.99 – 0.998, $p = 0.004$) (Table 2). For major postoperative complications, no independent association was found in multivariable analysis (OR 0.99, 95% CI 0.99 – 1.00, $p = 0.103$) (Table 3).

Table 3. Univariable and multivariable analyses on major postoperative complications (Clavien Dindo ≥3)

	Univariable			Multivariable		
	Odds Ratio	95% Confidence Interval	p-value	Odds Ratio	95% Confidence Interval	p-value
Gender – Male = reference	0.83	0.60 -1.16	0.283	0.85	0.6 – 1.20	0.354
Age (years)	1.02	1.01 – 1.04	0.009	1.01	1.00 – 1.03	0.142
Charlson comorbidity	1.13	1.02 – 1.25	0.017	1.08	0.97 – 1.20	0.187
Rectal carcinoma	1.54	1.09 – 2.17	0.014	1.72	1.19 – 2.47	0.004
Surgical technique - Open procedure = reference	0.47	0.34 – 0.66	<0.001	0.47	0.33 – 0.67	<0.001
Gross household income	0.99	0.99 – 0.998	0.009	0.99	0.99 – 1.00	0.103

SES and overall survival

OS increased gradually with increasing socioeconomic status (Figure 2). The median OS was only reached in Q1 (88.9 months, 95% CI 79.1-98.7), whereas the median OS was not reached in any of the other quartiles. Patients in Q1 had a worse OS compared with patients in Q2 (p = 0.016) and patients in Q2 had a worse survival compared with patients in Q3 (p = 0.01). Patients in Q3 and Q4 had a similar survival (p = 0.918).

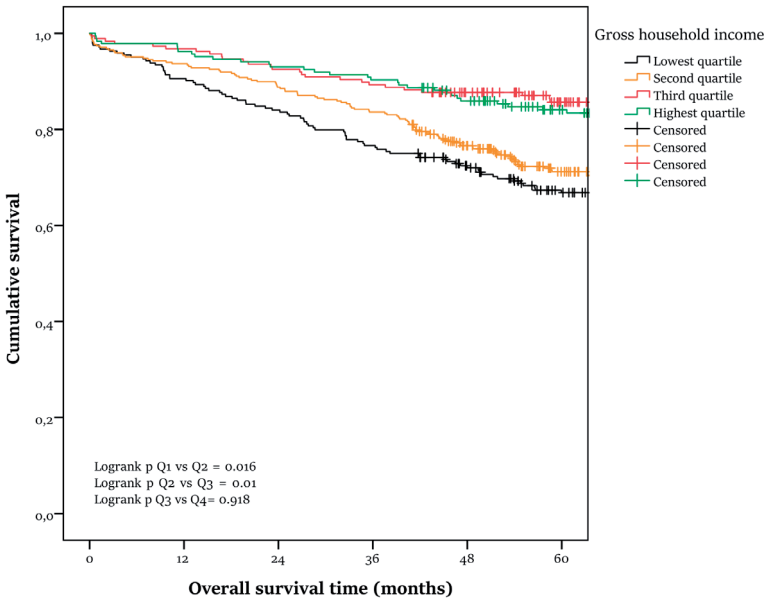

Figure 2. Overall 5 year survival per socioeconomic income quartile

In univariable analysis, GHI was associated with OS (HR 0.98, 95% CI 0.98 – 0.99, $p < 0.001$). Furthermore, age (HR 1.04, 95% CI 1.02 – 1.06, $p < 0.001$), CCI (HR 1.24, 95% CI 1.17 – 1.32, $p < 0.001$), tumour stage (HR 1.90, 95%CI 1.42-2.55, $p < 0.001$) and tumour grade (HR 1.89, 95%CI 1.29 – 2.76, $p = 0.001$) were associated with OS. In the multivariable analysis, GHI was not independently associated with OS (HR 0.99, 95%CI 0.99-1.00, $p = 0.158$), in contrast to age (HR 1.07, 95%CI 1.05-1.08, $p < 0.001$), CCI (HR 1.17, 95%CI 1.10-1.25, $p < 0.001$) tumour stage (HR 2.34, 95%CI 1.72-3.19, $p <0.001$) and tumour grade (HR 1.53, 95% CI 1.04-2.26, $p = 0.030$) (Table 4).

Table 4. Univariable and multivariable Cox regression analyses on overall survival

	Univariable			Multivariable		
	Hazard Ratio	95% Confidence Interval	p-value	Hazard Ratio	95% Confidence Interval	p-value
Age	1.04	1.02 – 1.06	<0.001	1.07	1.05 - 1.08	<0.001
Charlson comorbidity index	1.24	1.17 – 1.32	<0.001	1.17	1.10 - 1.25	<0.001
Tumour stage I (Ref)	-	-	-	-	-	-
Tumour stage II	1.26	0.93 – 1.71	0.138	1.20	0.88 - 1.65	0.253
Tumour stage III	1.90	1.42 – 2.55	<0.001	2.34	1.72 - 3.19	<0.001
Tumour grade good (Ref)	-	-	-	-	-	-
Tumour grade moderate	0.87	0.65 – 1.16	0.340	0.81	0.60 - 1.09	0.167
Tumour grade poor	1.89	1.29 – 2.76	0.001	1.53	1.04 - 2.26	0.030
Gross household income	0.98	0.98 – 0.99	<0.001	0.99	0.99 - 1.00	0.158

SES and cancer-specific survival

The median CSS was not reached for any of the quartiles. Patients in Q1 had a worse CSS compared with patients in Q2 ($p = 0.035$). No difference in survival was found between Q2 and Q3, nor for Q3 and Q4 ($p = 0.080$ and $p = 0.637$, respectively) (Figure 3).

In the univariable model for CSS, GHI showed a significant association (HR 0.98, 95%CI 0.98 – 0.99, $p < 0.001$) as well as age (HR 1.04, 95%CI 1.02 – 1.06, $p < 0.001$), CCI (HR 1.14, 95%CI 1.04 – 1.25, $p = 0.006$), tumour stage (HR 3.58, 95% CI 2.31 – 5.56, $p < 0.001$) and tumour grade (HR 2.62, 95% CI 1.52 – 4.52, $p = 0.001$). The association between GHI and CSS remained statistically significant in the multivariable model (HR 0.99, 95%CI 0.98-0.99, $p = 0.009$). Other factors that were independently associated with CSS were age (HR 1.03, 95%CI 1.01-1.05, $p = 0.001$) and tumour stage (HR 3.95, 95%CI 2.49-6.28, $p < 0.001$) (Table 5).

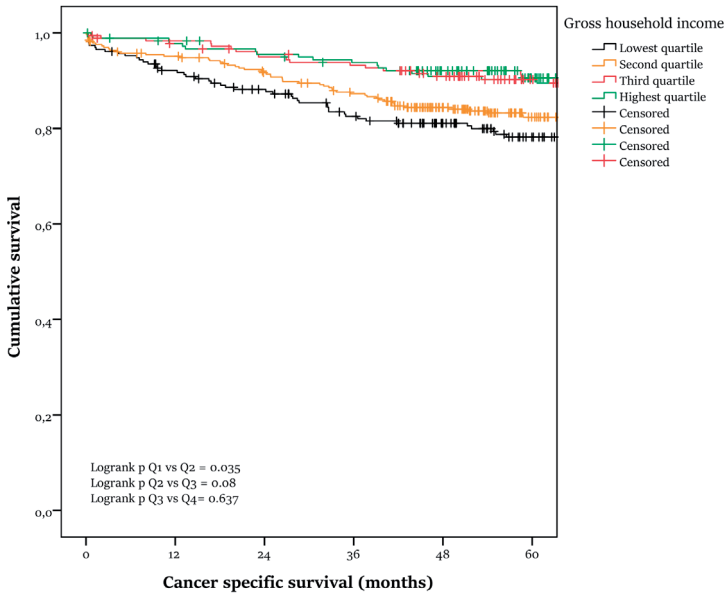

Figure 3. Cancer specific survival per socioeconomic income quartile

Table 5. Univariable and multivariable Cox regression analysis on cancer specific survival

	Univariable			Multivariable		
	Hazard Ratio	95% Confidence Interval	p-value	Hazard Ratio	95% Confidence Interval	p-value
Age	1.04	1.02 – 1.06	<0.001	1.03	1.01 – 1.05	0.001
Charlson comorbidity index	1.14	1.04 – 1.25	0.006	1.07	0.96 – 1.18	0.214
Tumour stage I (Ref)	-	-	-	-	-	-
Tumour stage II	1.33	0.81 – 2.19	0.262	1.28	0.76 – 2.16	0.353
Tumour stage III	3.58	2.31 – 5.56	<0.001	3.95	2.49 – 6.28	<0.001
Tumour grade good (Ref)	-	-	-	-	-	-
Tumour grade moderate	1.19	0.77 – 1.84	0.430	1.09	0.71 – 1.69	0.694
Tumour grade pore	2.62	1.52 – 4.52	0.001	1.84	1.06 – 3.19	0.031
Gross household income	0.98	0.98 – 0.99	<0.001	0.99	0.98 – 0.997	0.009

DISCUSSION

The results of this study show that lower SES is significantly associated with worse outcome in patients with stage I-III colorectal cancer undergoing curative surgery. We observed an increased rate of postoperative complications, after correcting for captured confounding factors. Although SES was not independently associated with OS, we observed a significant association between SES and CSS after correction for other known captured prognosticators (HR: 0.99 per percentile). This corresponds to a hazard ratio of 0.80 per quartile incremental SES increase. The current results should be viewed in the context of the Dutch equal access healthcare system. Therefore, the association between income and postoperative survival that was demonstrated in the present study cannot be attributed to inequality in healthcare resources. This is, to our knowledge, the first study to explore the influence of SES in a prospective cohort of consecutive colorectal cancer patients and stresses the importance of SES as a prognostic factor in these patients.

As in previous studies, our results indicate that SES is also associated with short-term outcome [17, 34]. This may partially be explained by confounding factors associated with both lower SES and higher postoperative morbidity. At baseline, diabetes mellitus was more prevalent in patients in the lower quartiles. This factor, as well as several others, such as liver disease, are incorporated in the ASA classification. These were significantly higher in the lower SES quartiles as well. Patients in the lower SES quartiles more often underwent open surgery, despite tumor characteristics being similar with regards to location, stage and pathologic prognostic factors. Moreover, patients were significantly older in the lower SES quartiles and therefor a larger part of these patients may have been retired the year prior to surgery and have less income.

These differences in treatment, without apparent clinical explanation, mirror those described in previous studies [34-36]. Even after correction for known confounding risk factors including comorbidities, SES was a significant predictor for postoperative morbidity. These results suggest that the correlation between SES and postoperative outcomes is determinant upon factors that are currently not adequately considered or understood.

In general, long-term results presented in our study are in line with previous studies, showing impaired survival in patients with low SES [20, 22, 23, 37]. Several previous studies have offered explanations for this survival discrepancy in a setting of equal healthcare access [38-51]. These possible explanations include psychological factors, medication compliance and diet, exercise, air pollution, and even epigenetic factors [38-51]. Interestingly, in our study, the correlation between SES and CSS was more outspoken than the correlation of SES with OS (i.e. all-cause mortality), making some of these explanations less plausible. In addition, this suggests that the survival discrepancy between SES quartiles was not solely attributable to the unequal distribu-

tion of age and comorbidities between the groups, as these factors have a less obvious effect on cancer related outcome. Fowler et al. showed that tumour stage and treatment contributed for a great part towards the difference in 3-month mortality between the most and least deprived patient groups [52]. Known and relevant tumour characteristics, as earlier described, did not significantly differ across SES quartiles. In contrast however, treatment strategies were different between groups. In addition to a different surgical approach between the quartiles, fewer patients of lower SES quartiles received neoadjuvant and adjuvant chemotherapy. The inequality of treatment with neo-adjuvant therapy can partially be explained by differences in proportion of patients with rectal cancer between the SES quartiles. Neoadjuvant chemotherapy is only registered in patients with rectal cancer whereas adjuvant chemotherapy is only offered to patients with colon cancer. However, as in our study, previous studies show that lower-middle-SES, and low-SES patients were less likely to receive chemotherapy in general [53]. Furthermore, since SES remained significantly associated with CSS after correction for baseline variables, it is possible that some of the driving factors of treatment differences cause additional differences in strategy not captured in our cohort. Besides differences in the administration of chemotherapy, compliance with treatment may also play a role. Prospective studies will be required to elucidate the exact causal mechanism of this remaining correlation.

GHI was chosen as a surrogate marker for SES in this study, as it is a proven accurate reflection of SES-related health disparities [26, 29-31]. It was not adjusted to household size, as previous studies showed this adjustment did not improve predictability of the associated health disparities [54]. Other reported determinants of SES include the highest attained level of education, current occupation, parent's education and occupation, and household conditions [34, 36, 55-57]. Unfortunately, no previous studies have compared the accuracy of these markers in a cohort of (colorectal) cancer patients, which limits their comparability [11-23]. Since GHI is a valid and readily available metric for all Dutch citizens registered by an independent organization (Dutch Statistics; CBS), we believe this marker adequately captures SES for our purposes. Use of this marker in different populations or different countries would add to the comparability of the plethora of SES studies currently being conducted.

Some inherent shortcomings of this study should be noted. First, with regards to patient factors, we were unable to determine several lifestyle factors of potential importance, due to the inherent limitations in our database. These include smoking, diet, and compliance with medication[58-60]. Even though these factors reportedly do not explain all difference, we believe correcting for them would have enhanced our results [30, 61, 62]. An additional drawback in our study was our inability to comment on exact healthcare consumption for CRC and comorbidities, limiting our ability of testing the premise of equal healthcare accessibility. Variation in SES between the hospitals can have some effect on the outcome, however, guideline adherence

was equal across the hospitals plus complications and survival outcomes did not differ between the hospitals.

Finally, ethnicity could be a confounding factor with SES, and the unfavorable surgical outcome of GHI in this study might also partially be explained by differences in ethnicity. However, ethnicity is not reported in the Netherlands, which limits this study because no analyses can be performed on the actual relevance of this risk factor[37].

In conclusion, our results show that low SES is associated with worse outcome in patients undergoing curative surgery for stage I-III colorectal cancer. Future studies are required to elucidate the association between SES and survival in cancer patients, which suggest that SES encompasses risk factors and behaviours currently not adequately considered. Such studies would require additional power and potential explaining factors and would ideally be able to distinguish between the effects of patient and treatment related measures.

REFERENCES

1. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, et al. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. *Eur J Cancer* 2013; 49(6):1374-403.
2. Corral J, Castells X, Molins E, et al. Long-term costs of colorectal cancer treatment in Spain. *BMC health services research* 2016; 16(1):56.
3. Dienstmann R, Mason MJ, Sinicrope FA, et al. Prediction of overall survival in stage II and III colon cancer beyond TNM system: a retrospective, pooled biomarker study. *Ann Oncol* 2017; 28(5):1023-1031.
4. Karahalios A, English DR, Simpson JA. Weight change and risk of colorectal cancer: a systematic review and meta-analysis. *Am J Epidemiol* 2015; 181(11):832-45.
5. Lauby-Secretan B, Scoccianti C, Loomis D, et al. Body Fatness and Cancer--Viewpoint of the IARC Working Group. *N Engl J Med* 2016; 375(8):794-8.
6. Johnson CM, Wei C, Ensor JE, et al. Meta-analyses of colorectal cancer risk factors. *Cancer Causes Control* 2013; 24(6):1207-22.
7. Yuhara H, Steinmaus C, Cohen SE, et al. Is diabetes mellitus an independent risk factor for colon cancer and rectal cancer? *Am J Gastroenterol* 2011; 106(11):1911-21; quiz 1922.
8. Newton S, Braithwaite D, Akinyemiju TF. Socio-economic status over the life course and obesity: Systematic review and meta-analysis. *PLoS ONE* 2017; 12(5):e0177151.
9. Pigeyre M, Rousseaux J, Trouiller P, et al. How obesity relates to socio-economic status: identification of eating behavior mediators. *Int J Obes (Lond)* 2016; 40(11):1794-1801.
10. Saydah S, Lochner K. Socioeconomic Status and Risk of Diabetes-Related Mortality in the U.S. *Public Health Reports* 2010; 125(3):377-388.
11. Lyratzopoulos G, Sheridan GF, Michie HR, et al. Absence of socioeconomic variation in survival from colorectal cancer in patients receiving surgical treatment in one health district: cohort study. *Colorectal Dis* 2004; 6(6):512-7.
12. Nur U, Rachet B, Parmar MK, et al. No socioeconomic inequalities in colorectal cancer survival within a randomised clinical trial. *Br J Cancer* 2008; 99(11):1923-8.
13. Coleman MP, Rachet B, Woods LM, et al. Trends and socioeconomic inequalities in cancer survival in England and Wales up to 2001. *Br J Cancer* 2004; 90(7):1367-73.
14. Schrijvers CT, Mackenbach JP. Cancer patient survival by socioeconomic status in seven countries: a review for six common cancer sites [corrected]. *J Epidemiol Community Health* 1994; 48(5):441-6.
15. Rachet B, Ellis L, Maringe C, et al. Socioeconomic inequalities in cancer survival in England after the NHS cancer plan. *Br J Cancer* 2010; 103(4):446-53.
16. Menvielle G, Rey G, Jougla E, et al. Diverging trends in educational inequalities in cancer mortality between men and women in the 2000s in France. *BMC Public Health* 2013; 13:823.
17. Moller H, Sandin F, Robinson D, et al. Colorectal cancer survival in socioeconomic groups in England: variation is mainly in the short term after diagnosis. *Eur J Cancer* 2012; 48(1):46-53.
18. Storli KE, Sondenaa K, Bukholm IR, et al. Overall survival after resection for colon cancer in a national cohort study was adversely affected by TNM stage, lymph node ratio, gender, and old age. *Int J Colorectal Dis* 2011; 26(10):1299-307.
19. Aarts MJ, Lemmens VE, Louwman MW, et al. Socioeconomic status and changing inequalities in colorectal cancer? A review of the associations with risk, treatment and outcome. *Eur J Cancer* 2010; 46(15):2681-95.

20. Manser CN, Bauerfeind P. Impact of socioeconomic status on incidence, mortality, and survival of colorectal cancer patients: a systematic review. *Gastrointest Endosc* 2014; 80(1):42-60 e9.
21. Egeberg R, Halkjaer J, Rottmann N, et al. Social inequality and incidence of and survival from cancers of the colon and rectum in a population-based study in Denmark, 1994-2003. *Eur J Cancer* 2008; 44(14):1978-88.
22. Breen N, Lewis DR, Gibson JT, et al. Assessing disparities in colorectal cancer mortality by socioeconomic status using new tools: health disparities calculator and socioeconomic quintiles. *Cancer Causes & Control* 2017; 28(2):117-125.
23. Le H, Ziogas A, Lipkin SM, et al. Effects of socioeconomic status and treatment disparities in colorectal cancer survival. *Cancer Epidemiol Biomarkers Prev* 2008; 17(8):1950-62.
24. Schoen C, Stremikis K. Mirror, mirror on the wall: how the performance of the US health care system compares internationally: Commonwealth Fund, 2010.
25. Schafer W, Kroneman M, Boerma W, et al. The Netherlands: health system review. *Health Syst Transit* 2010; 12(1):v-xxvii, 1-228.
26. Ultee KHJ, Tjeertes EKM, Bastos Gonçalves F, et al. The relation between household income and surgical outcome in the Dutch setting of equal access to and provision of healthcare. *PLOS ONE* 2018; 13(1):e0191464.
27. van Vugt JLA, Coebergh van den Braak RRJ, Lalmahomed ZS, et al. Impact of low skeletal muscle mass and density on short and long-term outcome after resection of stage I-III colorectal cancer. *Eur J Surg Oncol* 2018; 44(9):1354-1360.
28. Lalmahomed ZS, Coebergh van den Braak RRJ, Oomen MHA, et al. Multicenter fresh frozen tissue sampling in colorectal cancer: does the quality meet the standards for state of the art biomarker research? *Cell Tissue Bank* 2017; 18(3):425-431.
29. Sabanayagam C, Shankar A. Income is a stronger predictor of mortality than education in a national sample of US adults. *J Health Popul Nutr* 2012; 30(1):82-6.
30. Lantz PM, House JS, Lepkowski JM, et al. Socioeconomic factors, health behaviors, and mortality: results from a nationally representative prospective study of US adults. *Jama* 1998; 279(21):1703-8.
31. Geyer S, Peter R. Income, occupational position, qualification and health inequalities--competing risks? (comparing indicators of social status). *J Epidemiol Community Health* 2000; 54(4):299-305.
32. Wammes J JP, Westert G, Marit Tanke M. The Dutch Health Care System 2014. Available at: https://international.commonwealthfund.org/countries/netherlands/.
33. Dindo D, Demartines N, Clavien PA. Classification of surgical complications: a new proposal with evaluation in a cohort of 6336 patients and results of a survey. *Ann Surg* 2004; 240(2):205-13.
34. Cavalli-Bjorkman N, Lambe M, Eaker S, et al. Differences according to educational level in the management and survival of colorectal cancer in Sweden. *Eur J Cancer* 2011; 47(9):1398-406.
35. Robinson CN, Balentine CJ, Sansgiry S, et al. Disparities in the use of minimally invasive surgery for colorectal disease. *J Gastrointest Surg* 2012; 16(5):897-903; discussion 903-4.
36. Dik VK, Aarts MJ, Van Grevenstein WM, et al. Association between socioeconomic status, surgical treatment and mortality in patients with colorectal cancer. *Br J Surg* 2014; 101(9):1173-82.

37. Du XL, Meyer TE, Franzini L. Meta-analysis of racial disparities in survival in association with socioeconomic status among men and women with colon cancer. *Cancer* 2007; 109(11):2161-70.
38. Ultee KHJ, Bastos Gonçalves F, Hoeks SE, et al. Low Socioeconomic Status is an Independent Risk Factor for Survival After Abdominal Aortic Aneurysm Repair and Open Surgery for Peripheral Artery Disease. *European Journal of Vascular and Endovascular Surgery* 2015; 50(5):615-622.
39. Karen AM, Linda CG. Psychological Perspectives on Pathways Linking Socioeconomic Status and Physical Health. *Annual Review of Psychology* 2011; 62(1):501-530.
40. Steptoe A, Shankar A, Demakakos P, et al. Social isolation, loneliness, and all-cause mortality in older men and women. *Proceedings of the National Academy of Sciences* 2013; 110(15):5797-5801.
41. Ghali JK, Kadakia S, Cooper R, et al. Precipitating factors leading to decompensation of heart failure: Traits among urban blacks. *Archives of Internal Medicine* 1988; 148(9):2013-2016.
42. Artinian NT, Magnan M, Sloan M, et al. Self-care behaviors among patients with heart failure. *Heart & Lung: The Journal of Acute and Critical Care* 2002; 31(3):161-172.
43. Wilensky GR. From the health care financing administration. *JAMA* 1991; 266(24):3404-3404.
44. Wamala S, Merlo J, Bostrom G, et al. Socioeconomic disadvantage and primary non-adherence with medication in Sweden. *International Journal for Quality in Health Care* 2007; 19(3):134-140.
45. Rautio N, Jokelainen J, Oksa H, et al. Participation, socioeconomic status and group or individual counselling intervention in individuals at high risk for type 2 diabetes: One-year follow-up study of the FIN-D2D-project. *Primary Care Diabetes* 2012; 6(4):277-283.
46. Murray TC, Rodgers WM, Fraser SN. Exploring the relationship between socioeconomic status, control beliefs and exercise behavior: a multiple mediator model. *Journal of Behavioral Medicine* 2012; 35(1):63-73.
47. Wang DD, Leung CW, Li Y, et al. Trends in dietary quality among adults in the united states, 1999 through 2010. *JAMA Internal Medicine* 2014; 174(10):1587-1595.
48. Ricciuto LE, Tarasuk VS. An examination of income-related disparities in the nutritional quality of food selections among Canadian households from 1986–2001. *Social Science & Medicine* 2007; 64(1):186-198.
49. Roy M, Généreux M, Laverdière É, et al. Surveillance of Social and Geographic Inequalities in Housing-Related Issues: The Case of the Eastern Townships, Quebec (Canada). *International Journal of Environmental Research and Public Health* 2014; 11(5):4825.
50. Hajat A, Diez-Roux AV, Adar SD, et al. Air pollution and individual and neighborhood socioeconomic status: Evidence from the multi-ethnic study of atherosclerosis (MESA). *Environmental Health Perspectives* 2013; 121(11-12):1325-1333.
51. Saban KL, Mathews HL, de Von HA, et al. Epigenetics and social context: Implications for disparity in cardiovascular disease. *Aging and Disease* 2014; 5(5):346-355.
52. Fowler H, Belot A, Njagi EN, et al. Persistent inequalities in 90-day colon cancer mortality: an English cohort study. *Br J Cancer* 2017; 117(9):1396-1404.
53. Hines R, Markossian T, Johnson A, et al. Geographic residency status and census tract socioeconomic status as determinants of colorectal cancer outcomes. *Am J Public Health* 2014; 104(3):e63-71.

54. Lantz PM, House JS, Lepkowski JM, et al. Socioeconomic factors, health behaviors, and mortality: Results from a nationally representative prospective study of us adults. *JAMA* 1998; 279(21):1703-1708.
55. Galobardes B, Shaw M, Lawlor DA, et al. Indicators of socioeconomic position (part 1). *Journal of Epidemiology and Community Health* 2006; 60(1):7-12.
56. Smith G, Lynch J. Life course approaches to socioeconomic differentials in health. Oxford University Press, 2004.
57. Dedman DJ, Gunnell D, Davey Smith G, et al. Childhood housing conditions and later mortality in the Boyd Orr cohort. *J Epidemiol Community Health* 2001; 55(1):10-5.
58. Louwman WJ, Aarts MJ, Houterman S, et al. A 50% higher prevalence of life-shortening chronic conditions among cancer patients with low socioeconomic status. *Br J Cancer* 2010; 103(11):1742-8.
59. Wamala S, Merlo J, Bostrom G, et al. Socioeconomic disadvantage and primary non-adherence with medication in Sweden. *Int J Qual Health Care* 2007; 19(3):134-40.
60. Rautio N, Jokelainen J, Oksa H, et al. Participation, socioeconomic status and group or individual counselling intervention in individuals at high risk for type 2 diabetes: one-year follow-up study of the FIN-D2D-project. *Prim Care Diabetes* 2012; 6(4):277-83.
61. Braaten T, Weiderpass E, Lund E. Socioeconomic differences in cancer survival: the Norwegian Women and Cancer Study. *BMC Public Health* 2009; 9:178.
62. Pedrazzani C, Cerullo G, De Marco G, et al. Impact of age-related comorbidity on results of colorectal cancer surgery. *World J Gastroenterol* 2009; 15(45):5706-11.

Chapter 7

Circular RNAs in chemonaive lymph node negative colon cancer patients

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SIMPLE SUMMARY

Colon cancer (CC) is one of the most common types of cancer. Circular RNAs (circRNAs) appear to play an important role in tumor progression of CC. They are stably expressed in saliva, blood, and exosomes, potentially rendering them promising biomarkers for the diagnosis, prognosis, and treatment of CC. In this study we describe the identification of an extensive catalog of circRNAs in a large cohort of 181 chemonaive, stage I/II primary colon tumors and related circRNA expression to consensus molecular subtypes (CMS), microsatellite instability (MSI) status and clinical outcome. We observed that a high diversity in circRNAs was associated with favorable disease-free survival, and that several circRNAs were associated with MSI and CMS, demonstrating the potential clinical value of circRNAs in CC.

ABSTRACT

Introduction

Circular RNAs (circRNAs) appear important in tumor progression of colon cancer (CC). We identified an extensive catalog of circRNAs in 181 chemonaive stage I/II colon tumors, who underwent curative surgery between 2007 and 2014.

Methods

We identified circRNAs from RNAseq data, investigated common biology related to circRNA expression, and studied the association between circRNAs and relapse status, tumor stage, consensus molecular subtypes (CMS), tumor localization and microsatellite instability (MSI).

Results

We identified 2606 unique circRNAs. 277 circRNAs (derived from 260 genes) were repeatedly occurring in at least 20 patients of which 153 showed a poor or even negative ($R < 0.3$) correlation with the expression level of their linear gene. The circular junctions for circSATB2, circFGD6, circKMT2C and circPLEKHM3 were validated by Sanger sequencing. Multiple correspondence analysis showed that circRNAs were often co-expressed and that high diversity in circRNAs was associated with favorable disease-free survival (DFS), which was confirmed by Cox regression analysis (Hazard Ratio (HR) 0.60, 95% CI 0.38–0.97, $p = 0.036$). Considering individual circRNAs, absence of circMGA was significantly associated with relapse, whereas circSATB2, circNAB1, and circCEP192 were associated with both MSI and CMS.

Conclusion

This study represents a showcase of the potential clinical utility of circRNAs for prognostic stratification in patients with stage I-II colon cancer and demonstrated that high diversity in circRNAs is associated with favorable DFS.

INTRODUCTION

Colon cancer is one of the most common types of cancer with over 1 million new cases worldwide and around 9800 new cases in the Netherlands in 2018 [1]. Up to 21% of patients with stage I-II colon cancer and 40% of patients with stage III colon cancer will develop metastatic disease after curative surgery [2]. As much of the disparity in prognosis for clinically comparable patients remains unexplained, efforts are being directed at finding so far unknown factors that may play a role in the development and progression of colon cancer.

Transcriptome sequencing studies have identified many short and long RNAs with non-protein-coding ability [3-6]. These non-coding RNAs (ncRNAs) have received increasing attention in recent years due to their aberrant expression features associated with colorectal cancer (CRC) carcinogenesis [7-9]. Recent studies have shown that non-coding microRNAs can function as promising biomarkers for stage II [10, 11] and stage I-II colon cancer patients [12]. Circular RNAs (circRNAs) represent a re-discovered, abundant class of non-coding RNA molecules [13]. Altered expression of circRNAs is observed in cancer tissue compared to normal tissue [14-19], and particularly in CRC [20]. circRNA biogenesis derives from back-splicing, but the regulation and the frequency of this event are under investigation [20]. circRNAs form covalently closed, continuous loop structures produced through an end-to-end formation during transcription [21-23]. Increasing evidence shows that circRNAs can function as miRNA sponges, transcription regulators, and interfere with splicing, as well [20]. They are conserved, abundant and often exhibit tissue-, developmental-, and stage-specific expression [24, 25].

Due to their special circular structure, circRNAs are usually more stable than linear RNAs and are not easily degraded by exonucleases. They have been proven to remain stable in saliva, blood, and exosomes, which makes them promising biomarkers for the diagnosis, prognosis, and therapeutic assessment of cancer patients [26]. Recently, two novel circRNAs, both derived from the gene BCL2L12, were identified as biomarkers for stage II CRC patients [27]. Compared to conventional available cancer biomarkers (e.g., PSA and CEA), circRNAs are expected to have higher sensitivity and specificity in diagnosis and prognosis [28].

Taken together, these characteristics indicate that circRNAs could represent new clinical diagnostic and prognostic markers, and possibly provide new leads for the treatment of diseases. In this study we describe the identification of an extensive catalog of circRNAs in a large cohort of 181 chemonaive, stage I/II primary colon tumors and related these to tumor stage, localization, Consensus Molecular subtypes (CMS), microsatellite instability (MSI) status and clinical outcome.

METHODS

Study population and patient selection

Fresh-frozen tumor tissue was collected from 181 patients with stage I-II colon cancer undergoing curative surgery. These patients had been enrolled in the MATCH-study—a prospective multicenter cohort study in seven hospitals in the region of Rotterdam, the Netherlands-between 2007 and 2014. Patients have given informed consent on the storage and use of tissue samples, and the collection of clinical data for research purposes. The MATCH study was approved by the Erasmus MC IRB (MEC-2007-088). Inclusion criteria and additional clinical characteristics have been described [29].

Disease-free survival (DFS) was defined as the time elapsed between the date of surgery and either the date of any recurrence of disease or the date of the last follow-up visit at which a patient was considered to have no recurrence.

Sample collection and processing

Sample collection and processing, as well as RNA isolation and RNA sequencing have been described in detail previously [30-32]. All samples were reviewed by a pathologist (CHMvD) to ensure the presence of sufficient tumor cells (≥40%). Only samples with an RNA integrity number of at least 7.0 were selected for RNAseq analysis. RNA integrity numbers were assessed using the MultiNA Microchip Electrophoresis system (Shimadzu, Kyoto, Japan) [33].

Microsatellite instability

MSI analyses have previously been performed and described [32]. In short, the MSI analyses made use of the MSI Analysis System from Promega©, which is a fluorescent PCR-based assay for detection of microsatellite instability in seven markers, including five mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) and two pentanucleotide repeat markers (Penta C and Penta D) [34].

Consensus molecular subtypes

The CMS classification was performed using the "CMSclassifier" package (https://github.com/Sage-Bionetworks/CMSclassifier, accessed 23 may 2019), using the single-sample prediction parameter [35].

Identification of circRNAs

The methodology used to identify circRNA reads has previously been described in detail [36]. In short, the method developed by Smid et al. uses sequence reads that have a "secondary alignment" (SA) tag. When using paired-end sequence data, and assuming a circRNA molecule is present, the sequence read that aligns over the crossing of the junction would "point toward" its read-mate somewhere in the circle. Aligning these reads to the linear reference, the junction read will get an SA tag which will be assigned to two locations if and only if this is the one and unique alignment configuration the STAR software can find [37, 38]. The read-mate aligns somewhere in between these two locations. Finding additional read pairs showing this configuration with a breakpoint at the exact same location strengthens the evidence for circular transcripts. We included only regions with at least five reads crossing the circular junction. After filtering, GENCODE annotation was used to obtain the exon locations of genes that exactly matched to the circular region. For each sample, STAR also gives the raw read counts for all genes. These were normalized (Trimmed Mean of M-values implemented in edgeR [39]), and the normalized read counts were used to correlate with the number of junction reads of the circular transcripts. The script is also available at https://bitbucket.org/snippets/MSmid/Le949d/identify-circularrna-reads (uploaded 30 Oct 2018).

Multiple correspondence analysis (MCA)

For a substantial number of genes, only a linear transcript is detected in the majority of samples, which results in many missing values per circRNA. This in turn, complicates the use of standard cluster analysis for the identification of sample groups with similar circRNA-related biology. Therefore, circRNA data were considered categorical, i.e., a circRNA was scored as either "present" or "absent" in a sample. These categorical data are suitable for a multiple correspondence analysis (MCA), which is a generalized principle component analysis. An MCA generates a combined plot that shows both patients and circRNAs in such a way that patients and circRNAs that have similar patterns are closer together. Thus, the colon cancer tumor samples and circRNAs are projected onto the same plane, in which the relative distance to either the samples or the circRNAs is meaningful. The 0,0 point corresponds to a sample or circRNA with an average profile. The R-package "ade4" was used to perform the MCA in R version 3.4.1. Custom functions to plot the MCA results are available upon request of the authors.

Reverse transcription, quantitative PCR, and sanger sequencing

Candidate circular RNAs were selected, and divergent primers were designed that are only able to amplify and detect the circular and not the corresponding linear mRNA (Table S1). Total RNA, isolated with RNA-Bee according to the manufacturer's instructions (CS105B, TEL TEST), was reverse transcribed into cDNA with the H-minus RevertAid First Strand cDNA Synthesis Kit

(K1632, ThermoFisher Scientific, Waltham, MA, USA), followed by an RNAse-H step (AM2293; Ambion). For Sanger sequencing, cDNA from five individual patient samples were used for PCR. cDNA was amplified for 35 cycles using Phusion high-fidelity DNA polymerase (ThermoFisher Scientific) in a total reaction volume of 25 µL, containing 400 nM of each primer and 160 µM dNTPs. For every circRNA two resulting PCR amplicons were purified from gel using the Qiaquick Gel Extraction Kit from Qiagen (Hilden, Germany) according to the manufacturer's protocol and subjected to Quick Shot Sanger Sequencing by BaseClear BV (Leiden, The Netherlands).

Statistical analyses

As indicated above for a substantial number of genes, only a linear transcript was detected in the majority of samples, which results in many missing values per circRNA. This hampers statistical analyses of circRNA expression levels and therefore we categorized the circRNA data into "present" or "absent" for statistical evaluation as well. We used circRNAs present in at least 20 samples to ensure a sufficient number of events for subsequent statistical analyses. STATA version 14 and SPSS Version 24.0 (SPSS, Inc., Chicago, IL, USA) were used to perform the statistical tests that are also indicated in the text. Cox's proportional-hazards regression was used to evaluate the (log-transformed) number of uniquely present circRNAs per sample, hereafter called circRNA diversity, with DFS, or as "present"/"absent" when evaluating individual circRNAs. Survival curves were evaluated using the logrank test (for individual circRNAs) or with the logrank test for trend (for circRNA diversity, after dividing into three equal quantiles). Pearson's correlation was used to correlate the circRNA expression with the expression of the linear gene it was derived from. Analyses between categorical variables (like present/absent of a circRNA versus MSI yes/no) were analyzed using Fisher's exact test. Reported p-values are two-sided and considered significant at $p \leq 0.05$. p-values were corrected for multiple testing using Benjamini–Hochberg's FDR correction when evaluating multiple circRNAs, which were considered significant at $p < 0.10$.

RESULTS

CircRNA expression in colon cancer

We analyzed RNAseq data of 181 patients with chemonaive, stage I/II primary colon cancer. The median follow-up time was 53 months (IQR 37–59). Clinical and histopathological characteristics are listed in Table 1.

Circular RNAs were defined as present when at least five reads crossed the circular junction [36]. This resulted in the identification of 2606 distinct circRNAs in the entire cohort, of which 1860 were derived from known genes. Sixty-three percent of these were repeatedly occurring

Table 1. Clinical and histopathological characteristics.

Clinical variables	Categories	N = 181	%
Gender	Female	92	(50.9)
	Male	89	(49.2)
Age (median, IQR)		70	(63–76)
Tumor stage	Stage I	66	(36.5)
	Stage II	115	(63.5)
T status	T2	66	(36.5)
	T3	110	(60.8)
	T4	5	(2.8)
Nodal status	N0 ≥ 10 nodes assessed	149	(82.2)
	N0 < 10 nodes assessed	32	(17.3)
Tumor grade	Good	16	(8.8)
	Poor	10	(5.5)
	Moderate	152	(84)
	Unknown	3	(1.7)
Location	Right	92	(50.8)
	Left	89	(49.2)
MSI status	MSI	44	(24.3)
	MSS	137	(75.7)
Relapse	No	152	(84)
	Yes	29	(16)

Abbreviations: MSI = Microsatellite instability, MSS = Microsatellite stable.

in at least two colon cancer samples (*n* = 1172) (Figure 1), whereas 277 (15%) were observed in 20 samples or more (Table S2). The most repeatedly occurring circRNAs were derived from SMARCA5, HIPK3, ZKSCAN1 and FBXW7 and were observed in 177 samples each (n.b. not the same 177 samples for all four circRNAs) (Table 2). For 29 genes we observed that more than one unique circRNA was derived from the linear sequence (Table S2). Relative to the total number of samples expressing at least one circRNA from the respective gene, varying levels of co-expression between circRNAs derived from the same gene were observed with a median of 26.19% (range: 4.40–82.30%).

We correlated the number of circRNA reads per circRNA with the expression of the linear gene from which the circRNA was derived. To avoid possible spurious correlations, only the 277 circRNAs found in at least 20 samples were analyzed. The vast majority of circRNAs showed a positive correlation with the linear gene from which the circRNA is derived (Figure 2). However, this correlation was poor ($R < 0.3$) for 126 circRNAs. Twenty-seven circRNAs showed a negative correlation with their corresponding linear gene, suggesting the circRNA may function independently from the linear transcript.

We randomly selected four circRNAs representative of the entire list of identified circRNAs to get an unbiased validation of our circRNA identification pipeline. Our four candidates include two from the top-20 circRNAs showing the most positive and negative correlations to their linear counterparts respectively (SATB2_chr2:199368605-199433515, R = 0.61 and PLEKHM3_chr2:207976651-207977587, R = –0.17), and one novel circRNA (FGD6

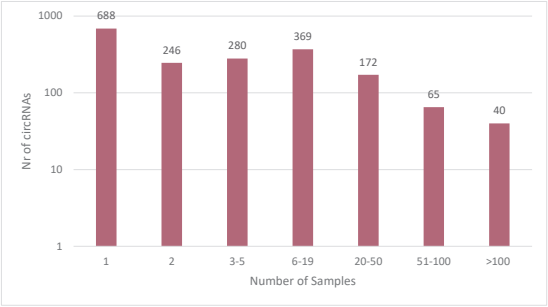

Figure 1. Histogram showing the distribution of circRNA occurrence in the 181 stage I/II colon cancer samples. A total of 1860 distinct circRNAs were identified which were derived from known genes.

Table 2. Most frequently recurring circRNAs.

Circular Region	Ensembl Gene ID	Gene	Exons	Nr. of Samples	R *
chr4:143543509-143543973	ENSG00000153147	*SMARCA5*	15-16	177	0.296
chr11:33286413-33287512	ENSG00000110422	*HIPK3*	2	177	0.541
chr7:100023419-100024308	ENSG00000106261	*ZKSCAN1*	2-3	177	0.539
chr4:152411303-152412530	ENSG00000109670	*FBXW7*	2	177	0.437

* R indicates the Pearson correlation between the number of circRNA reads and mRNA reads for that gene.

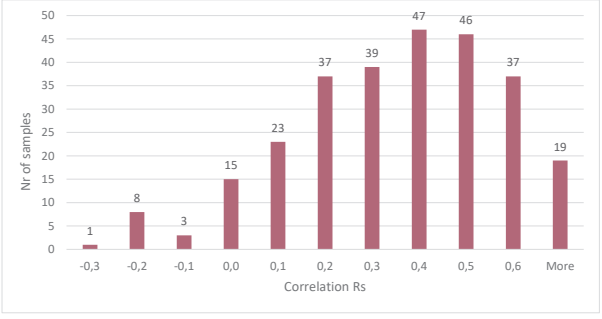

Figure 2. Histogram showing the distribution of the observed correlations between the number of circRNA and mRNA reads per gene.

(chr12:95208843-95211268), $R = 0.39$) not present in circBase (circbase.org [40]). The identified junctions in the RNAseq data were verified for all four selected circRNAs by Sanger sequencing, thereby demonstrating the validity of our circRNA identification algorithm [36] (Figure 3).

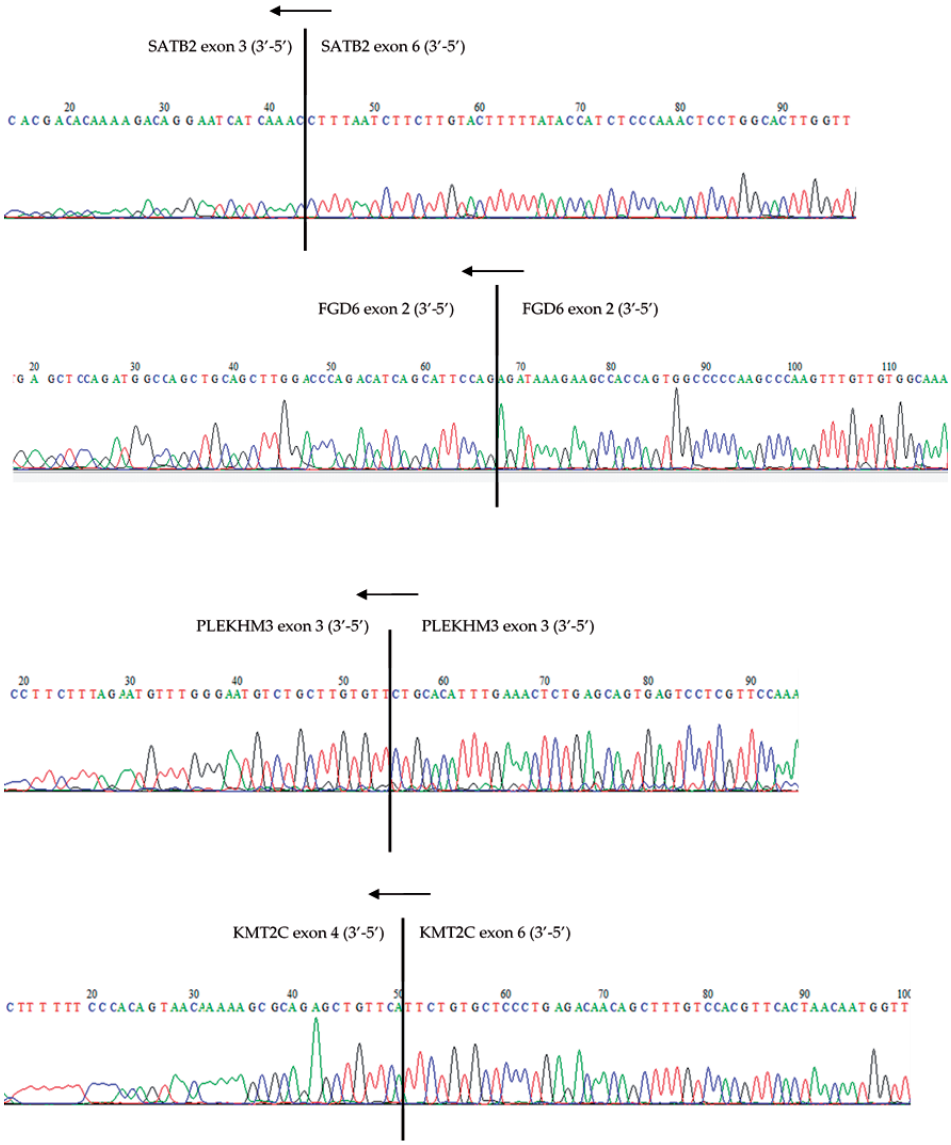

Figure 3. Sanger sequencing was used to validate the circular exon junctions of circSATB2, circKMT2C, circFGD6, and circPLEKHM3 identified from the RNAseq data.

CircRNA expression patterns are associated with relevant clinical factors

For a substantial number of genes, only a linear transcript is detected in the majority of samples, which results in many missing values per circRNA. This in turn complicates the use of standard cluster analysis for the identification of circRNA/sample groups with similar circRNA-related biology. To be able to investigate circRNA profiles, we categorized circRNAs as ”present” or “absent” in a sample and used this in a multiple correspondence analysis (MCA) to find naturally occurring subgroups. An MCA plot projects the colon tumor samples and circRNAs onto the same plane, in which the relative distance to either the samples or the circRNAs is meaningful. As such, samples that group close together have more similar circRNA profiles. In addition, since circRNAs have two states (present/absent), both these states are used in the analysis. Thus, two circRNAs that are ”present” frequently in the same samples (co-occurrence) will be placed at a short distance, but this is also true for circRNAs that are mutually exclusive (presence of a circRNA and absence of the other circRNA) across the samples. Coloring the circRNA states will reveal the co-occurrence/mutual-exclusivity.

For the MCA analysis, we used the 277 repeatedly occurring circRNAs (i.e., those which are present in at least 20 samples) and labelled these in each sample as “present” or ”absent” as defined above. After MCA analysis, we first colored genes based on the circRNA state (Figure 4a). As shown by the clear separation of the “present” and “absent” state, circRNAs do not show mutual-exclusivity (which would show up as a red triangle among blue triangles or vice versa), but rather are often co-expressed in the same samples. Furthermore, the variability (spread among the *x*-axis) of the “present” profiles indicates different circRNA expression profiles are present among the samples, or, in other words, samples show a large diversity of expressed circRNAs.

Next, we colored samples according to relapse status (Figure 4b), tumor stage (Figure 4c), CMS (Figure 4d), tumor localization (Figure 4e) and MSI (Figure 4f). Patients showing relapse (1) (Figure 4b) have profiles that are close to the “absent” group (group without circRNAs), which indicates that few genes give rise to circRNA expression in these samples. Indeed, when analyzing circRNA diversity (the number of distinct circRNA molecules in a sample) we found that a high diversity in circRNAs is associated with a favorable DFS: Cox regression using circRNA diversity as (log-transformed) continuous variable: Hazard Ratio (HR) 0.60, 95% CI 0.38–0.97, $p = 0.036$. Figure 5 shows Kaplan–Meier curves in which the levels of diversity of circRNAs were split into three equal quantiles to visualize the association between circRNA-diversity and DFS. The difference in DFS between the three quantiles was evaluated using the logrank test for trend, to account for the ordered structure of the sample groups (high, intermediate and low circRNA diversity; $p = 0.050$). High diversity was not associated with other clinical factors such as tumor stage, tumor side, MSI status, or CMS (diversity as continuous variable).

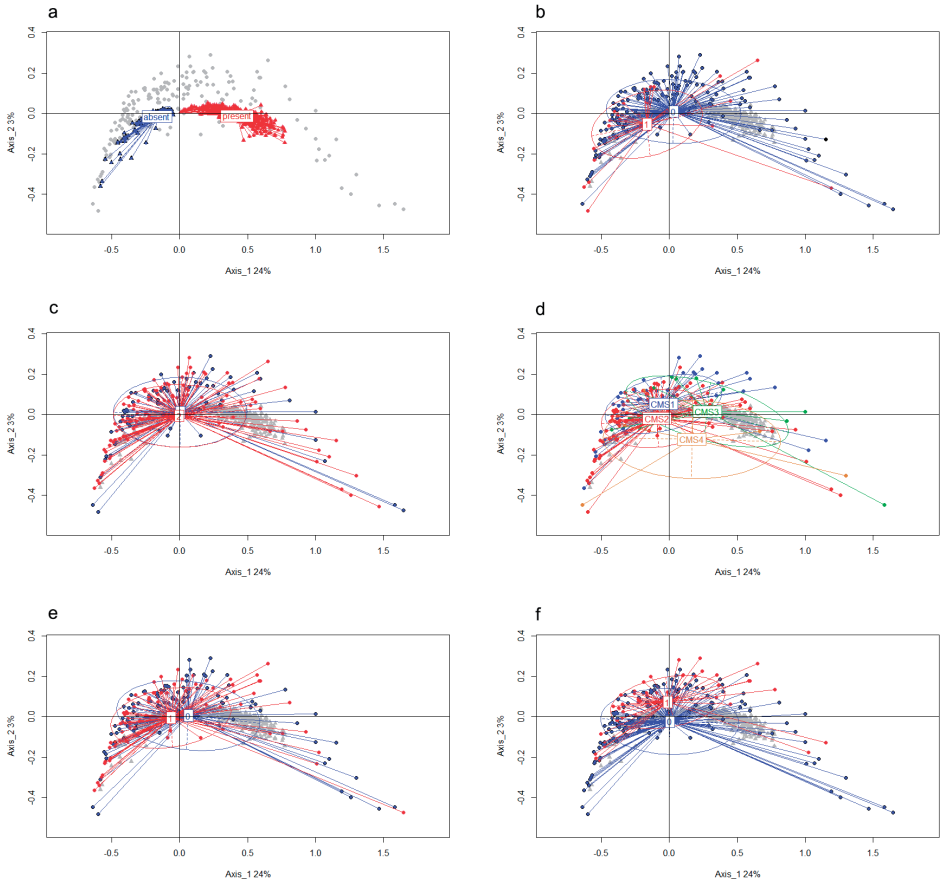

Figure 4. MCA analysis plots in which samples (closed circles) and circRNAs (triangles) are projected onto the same plane. (**a**). Blue and red indicate circRNAs without (absent) or with (present) circRNA expression, where similarity of two circRNA expression profiles (either both present (both red), both absent (both blue), or mutually exclusive (one red and one blue)) over the samples results in a small relative distance between these circRNAs. (**b**). Samples are colored based on relapse status, red indicates patients who relapse (1) showing circRNA profiles that are close to the "circRNA absent" group. (**c**). Samples are colored based on tumor stage I (blue) or stage II (red) showing no clear distinction in circRNA profiles. (**d**). The consensus molecular subtype (CMS) of the samples are indicated. CMS3 samples are most closely located to the "circRNA present" group. (**e**). Samples colored based on tumor side (left = 0; in blue, right = 1; in red) and (**f**), based on microsatellite instability (MSI) (MSI = 1; in red, MSS = 0; in blue).

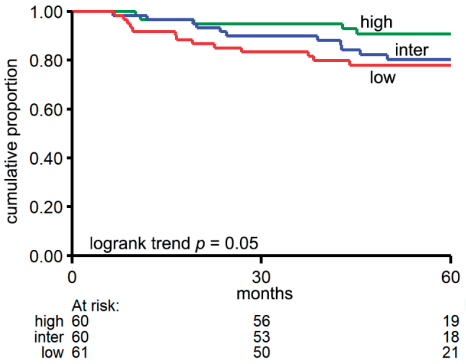

Figure 5. Kaplan–Meier survival curves of disease free survival in which patients were grouped in 3 equal quantiles based on their diversity in circRNA expression. The red line represents the quantile with the lowest diversity in circRNAs, the blue line represents the quantile with intermediate diversity in circRNAs and the green line represents the quantile with the highest diversity in circRNAs.

The MCA plot of tumor side (Figure 4e) shows that right-sided tumors are closer to the absent group (group of samples without circRNAs) than the left-sided tumors –therefore also closer to the relapse group, but this association was not significant. Combining CMS grouping (Figure 4d) and MSI (Figure 4f) shows that, as expected, samples that are CMS1 and those that are MSI tumors have a similar position. Combining CMS (Figure 4d) and circRNA diversity (Figure 4a) leads to the conclusion that CMS3 patients have the highest diversity in circRNAs, and CMS2 patients the lowest. As to tumor stage, there was no clear distinction between stage I and II tumors with regard to circRNAs (Figure 4e).

Next to this global analysis of overall circRNA profiles in the samples, we also investigated whether the presence/absence of specific circRNAs was associated with relapse status, tumor stage, CMS, tumor localization, and MSI. Whereas no specific circRNAs were significantly associated with tumor stage and localization, the presence/absence of nine circRNAs was associated with CMS and five with MSI (Table 3; Fisher exact test $p < 0.0003$; Benjamini-Hochberg corrected p-value < 0.1). circSATB2 (Special AT-rich sequence-binding protein 2), circNAP1 (Nucleosome assembly protein) and circCEP192 (Centrosomal Protein 192) each correlated with both MSI and CMS. Only absence of circMGA (MAX dimerization protein) was significantly associated with relapse (Table 3; Fisher exact test $p = 0.0002$; Benjamini–Hochberg corrected p-value = 0.06). Kaplan-Meier analysis showed that patients in whom circMGA was detected ($n = 94$) had a favorable DFS compared to patients in which circMGA was not detected ($n = 87$; log-rank test $p < 0.001$, Cox HR 0.22 95%CI 0.09–0.53, $p < 0.001$) (Figure 6).

Table 3. Association of presence/absence of circRNAs with relapse status, tumor stage, CMS and MSI.

Name	Ensemble Gene ID	Circular Region	Fisher p-Value	FDR *	Comparison
circSATB2	ENSG00000119042	chr2:199368605-199433515	2.47×10^{-8}	6.84×10^{-6}	MSI
			2.41×10^{-8}	6.68×10^{-6}	CMS
circNAB1	ENSG00000138386	chr2:190659158-190673153	6.48×10^{-7}	0.000179	MSI
			0.00007584	0.02078	CMS
circZBTB44	ENSG00000196323	chr11:130260856-130261930	3.4×10^{-5}	0.009347	MSI
circCEP192	ENSG00000101639	chr18:12999421-13019207	0.000152	0.041758	MSI
			0.00007808	0.021316	CMS
		chr18:12999421-13030609	0.000233	0.063043	CMS
circUBAP2	ENSG00000137073	chr9:33960826-33973238	0.000238	0.064846	MSI
circMGA	ENSG00000174197	chr15:41668828-41669959	0.000235	0.064979	DFSI
circASPH	ENSG00000198363	chr8:61618978-61653661	1.19×10^{-7}	3.28×10^{-5}	CMS
circCCSER2	ENSG00000107771	chr10:84438512-84477665	3.9×10^{-5}	0.010728	CMS
circZNF609	ENSG00000180357	chr15:64499293-64500167	0.00017	0.046128	CMS
circFUT8	ENSG00000033170	chr14:65561337-65561767	0.00026	0.070135	CMS
circMRPS35	ENSG00000061794	chr12:27714780-27724187	0.000299	0.08035	CMS

* False discovery rate (Benjamini–Hochberg procedure). Abbreviations: MSI = microsatellite instability, CMS = consensus molecular subtype.

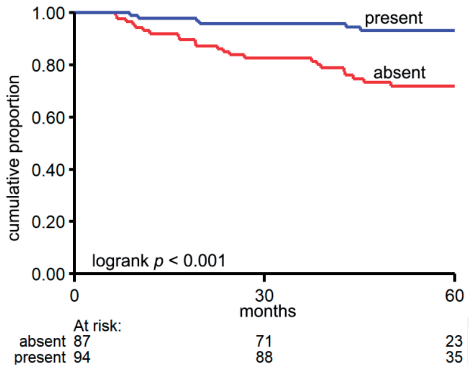

Figure 6. Kaplan–Meier analysis of patients in whom circMGA was detected (n = 94) versus patients in whom circMGA was not detected (n = 87).

DISCUSSION

With the use of RNAseq data, we could establish the presence of a wide variety of circRNAs in chemonaive lymph node negative, stage I/II primary colon tumors. Previous studies have been limited by the small number of circRNAs screened, the small sample size and retrospective data. Our study, however, concerned 181 patients included in a prospective, multicenter co-

hort study, and is therefore, to our knowledge, the largest circRNA-based biomarker discovery study done in stage I/II colon cancer.

The four most repeatedly occurring circRNAs that we found (177/181 samples), circSMARCA5, circHIPK3, circFBXW7 and circZKSCAN1, have also been described as such in previous studies. circSMARCA5 was reported to be induced during epithelial-to-mesenchymal transition, which is an important mechanism during the metastatic process that has been associated with the pathogenesis of several cancers [26, 41-44]. circHIPK3 has been described to promote CRC growth and metastasis by sponging miR-7 [45]. Furthermore, previous research in CRC cell lines showed that circFBXW7 is conducive in controlling the progression of CRC through NEK2, mTOR, and PTEN signaling pathways [37]. The correspondence of our finding with previous results clearly underlines the validity of our approach in identifying circRNAs. In addition, we performed Sanger sequencing to verify four randomly selected circRNAs (circSATB2, circKMT2C circFGD6, and circPLEKHM3) and successfully validated the identified circular junctions for all four circRNAs.

In the studied cohort of chemonaive lymph node negative colon cancer patients, a first highlight was the finding that high diversity of circRNAs present in colon cancer tissue was associated with favorable DFS. Vo et al showed that across different cancer types, total circRNA abundance was lower in cancer compared to normal tissue, suggesting that the reduction of circRNA generation could be associated with loss of cellular differentiation [46]. More specifically, presence of circMGA was significantly associated with a favorable DFS. Together, these findings support the idea that circRNAs might play a functional role in cancer metastasis [26]. Recent studies provide evidence for a tumor suppressive role for the gene MGA (MAX dimerization protein) in colorectal cancer [47]. In lung adenocarcinoma, the molecular function of MGA appears to be antagonistic to that of MYC. To our knowledge, this is the first study associating the circRNA emanating from this gene with colon cancer or any other malignancies.

A second highlight of this study is the association between circRNAs and distinct colorectal cancer subtypes. Presence/absence of nine and five circRNAs was significantly associated with CMS and MSI, respectively, of which circSATB2, circNAB1, circCEP192 were overlapping. Although we were unable to find a suitable publicly available RNAseq dataset to validate the associations we found between circRNAs and clinical parameters in our cohort of stage I-II colon cancer patients, a number of the circRNAs we found to be associated with distinct subtypes of colon cancer were described before in cancer. circSATB2 has been described to play a notable role in the progression of lung cancer by binding to miR-326 [48], which in turn is associated with CRC [49]. The association between CEP192, NAB1 and CRC or other cancers, has not been described in previous studies. A role in CRC was proven for circZNF609 (Zinc Finger Protein 609), which is down-regulated in CRC tissue and promotes apoptosis in CRC by upregulating

p53 [50]. circUBAP2 (ubiquitin associated protein 2) facilitates CRC progression by sponging miR-199a to upregulate VEGFA which implies that circUBAP2 may be a potential therapeutic biomarker for CRC [51]. Furthermore, circZBTB44 (Zinc Finger and BTB Domain Containing 44) and circZNF609 are both upregulated in acute lymphoblastic leukemia [52] of which especially circZNF609 has a known oncogenic potential in multiple other cancers as well [53-57]. CircASPH (Aspartate Beta-Hydroxylase) expression is upregulated in lung adenocarcinoma [58] and, finally, circFUT8 (Fucosyltransferase 8) functions as a tumor suppressor in bladder cancer cells where low circFUT8 was associated with poor prognosis, high histological grade, and lymph node metastasis [59]. The largest strength of this study is its prospective, multicenter study design and that it is, to our knowledge, the largest circRNA-based biomarker discovery study performed in stage I/II colon cancer. However, as mentioned before, a limitation of this study is that we were unable to find a suitable publicly available dataset to validate the associations we found between circRNAs and clinical parameters. Furthermore, some of the subgroup analyses, such as MSI, resulted in rather small sample sizes in outcome, increasing the chance of type II errors.

In conclusion, this study generated a comprehensive catalog of circRNAs in colon cancer and demonstrated the potential biological and clinical relevance of circRNAs in patients with stage I-II colon cancer. We demonstrated high diversity in circRNAs is associated with favorable DFS. As such, circRNAs represent a promising addition to the biomarker repertoire for colon cancer.

REFERENCES

1. Cijfers over kanker. Available at: www.cijfersoverkanker.nl.
2. Sargent DJ, Patiyil S, Yothers G, et al. End points for colon cancer adjuvant trials: observations and recommendations based on individual patient data from 20,898 patients enrolled onto 18 randomized trials from the ACCENT Group. *J Clin Oncol* 2007; 25(29):4569-74.
3. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* 2009; 10(1):57-63.
4. Iyer MK, Niknafs YS, Malik R, et al. The landscape of long noncoding RNAs in the human transcriptome. *Nat Genet* 2015; 47(3):199-208.
5. Muers M. RNA: Genome-wide views of long non-coding RNAs. *Nat Rev Genet* 2011; 12(11):742.
6. Sanger HL, Klotz G, Riesner D, et al. Viroids are single-stranded covalently closed circular RNA molecules existing as highly base-paired rod-like structures. *Proc Natl Acad Sci U S A* 1976; 73(11):3852-6.
7. Yan B, Gu W, Yang Z, et al. Downregulation of a long noncoding RNA-ncRuPAR contributes to tumor inhibition in colorectal cancer. *Tumour Biol* 2014; 35(11):11329-35.
8. Cao Y, Lin M, Bu Y, et al. p53-inducible long non-coding RNA PICART1 mediates cancer cell proliferation and migration. *Int J Oncol* 2017; 50(5):1671-1682.
9. Xu J, Zhang R, Zhao J. The Novel Long Noncoding RNA TUSC7 Inhibits Proliferation by Sponging MiR-211 in Colorectal Cancer. *Cell Physiol Biochem* 2017; 41(2):635-644.
10. Jacob H, Stanisavljevic L, Storli KE, et al. A four-microRNA classifier as a novel prognostic marker for tumor recurrence in stage II colon cancer. *Sci Rep* 2018; 8(1):6157.
11. Bullock MD, Pickard K, Mitter R, et al. Stratifying risk of recurrence in stage II colorectal cancer using deregulated stromal and epithelial microRNAs. *Oncotarget* 2015; 6(9):7262-79.
12. Grassi A, Perilli L, Albertoni L, et al. A coordinate deregulation of microRNAs expressed in mucosa adjacent to tumor predicts relapse after resection in localized colon cancer. *Mol Cancer* 2018; 17(1):17.
13. Su M, Xiao Y, Ma J, et al. Circular RNAs in Cancer: emerging functions in hallmarks, stemness, resistance and roles as potential biomarkers. *Mol Cancer* 2019; 18(1):90.
14. Memczak S, Jens M, Elefsinioti A, et al. Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* 2013; 495(7441):333-8.
15. Zhang Y, Zhang XO, Chen T, et al. Circular intronic long noncoding RNAs. *Mol Cell* 2013; 51(6):792-806.
16. Ashwal-Fluss R, Meyer M, Pamudurti NR, et al. circRNA biogenesis competes with pre-mRNA splicing. *Mol Cell* 2014; 56(1):55-66.
17. Hansen TB, Jensen TI, Clausen BH, et al. Natural RNA circles function as efficient microRNA sponges. *Nature* 2013; 495(7441):384-8.
18. Qu S, Yang X, Li X, et al. Circular RNA: A new star of noncoding RNAs. *Cancer Lett* 2015; 365(2):141-8.
19. Zhang L. Circular RNA: The main regulator of energy metabolic reprogramming in cancer cells. *Thorac Cancer* 2020; 11(1):6-7.
20. Artemaki PI, Scorilas A, Kontos CK. Circular RNAs: A New Piece in the Colorectal Cancer Puzzle. *Cancers (Basel)* 2020; 12(9).
21. Hsu MT, Coca-Prados M. Electron microscopic evidence for the circular form of RNA in the cytoplasm of eukaryotic cells. *Nature* 1979; 280(5720):339-40.

22. Wilusz JE, Sharp PA. Molecular biology. A circuitous route to noncoding RNA. *Science* 2013; 340(6131):440-1.
23. Cocquerelle C, Mascrez B, Hetuin D, et al. Mis-splicing yields circular RNA molecules. *FASEB J* 1993; 7(1):155-60.
24. Salzman J, Chen RE, Olsen MN, et al. Cell-type specific features of circular RNA expression. *PLoS Genet* 2013; 9(9):e1003777.
25. Jeck WR, Sorrentino JA, Wang K, et al. Circular RNAs are abundant, conserved, and associated with ALU repeats. *RNA* 2013; 19(2):141-57.
26. Zhang M, Xin Y. Circular RNAs: a new frontier for cancer diagnosis and therapy. *J Hematol Oncol* 2018; 11(1):21.
27. Karousi P, Artemaki PI, Sotiropoulou CD, et al. Identification of Two Novel Circular RNAs Deriving from BCL2L12 and Investigation of Their Potential Value as a Molecular Signature in Colorectal Cancer. *Int J Mol Sci* 2020; 21(22).
28. Wang S, Zhang K, Tan S, et al. Circular RNAs in body fluids as cancer biomarkers: the new frontier of liquid biopsies. *Mol Cancer* 2021; 20(1):13.
29. van Vugt JLA, Coebergh van den Braak RRJ, Lalmahomed ZS, et al. Impact of low skeletal muscle mass and density on short and long-term outcome after resection of stage I-III colorectal cancer. *Eur J Surg Oncol* 2018; 44(9):1354-1360.
30. Sieuwerts AM, Lyng MB, Meijer-van Gelder ME, et al. Evaluation of the ability of adjuvant tamoxifen-benefit gene signatures to predict outcome of hormone-naive estrogen receptor-positive breast cancer patients treated with tamoxifen in the advanced setting. *Mol Oncol* 2014; 8(8):1679-89.
31. Sieuwerts AM, Meijer-van Gelder ME, Timmermans M, et al. How ADAM-9 and ADAM-11 differentially from estrogen receptor predict response to tamoxifen treatment in patients with recurrent breast cancer: a retrospective study. *Clin Cancer Res* 2005; 11(20):7311-21.
32. Coebergh van den Braak RRJ, Sieuwerts AM, Kandimalla R, et al. High mRNA expression of splice variant SYK short correlates with hepatic disease progression in chemonaive lymph node negative colon cancer patients. *PLoS One* 2017; 12(9):e0185607.
33. Smid M, Coebergh van den Braak RRJ, van de Werken HJG, et al. Gene length corrected trimmed mean of M-values (GeTMM) processing of RNA-seq data performs similarly in intersample analyses while improving intrasample comparisons. *BMC Bioinformatics* 2018; 19(1):236.
34. Bacher JW, Flanagan LA, Smalley RL, et al. Development of a fluorescent multiplex assay for detection of MSI-High tumors. *Dis Markers* 2004; 20(4-5):237-50.
35. Guinney J, Dienstmann R, Wang X, et al. The consensus molecular subtypes of colorectal cancer. *Nat Med* 2015; 21(11):1350-6.
36. Smid M, Wilting SM, Uhr K, et al. The circular RNome of primary breast cancer. *Genome Res* 2019; 29(3):356-366.
37. Lu H, Yao B, Wen X, et al. FBXW7 circular RNA regulates proliferation, migration and invasion of colorectal carcinoma through NEK2, mTOR, and PTEN signaling pathways in vitro and in vivo. *BMC Cancer* 2019; 19(1):918.
38. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 2013; 29(1):15-21.
39. Robinson MD, Oshlack A. A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol* 2010; 11(3):R25.
40. Glazar P, Papavasileiou P, Rajewsky N. circBase: a database for circular RNAs. *RNA* 2014; 20(11):1666-70.

41. Gurzu S, Turdean S, Kovecsi A, et al. Epithelial-mesenchymal, mesenchymal-epithelial, and endothelial-mesenchymal transitions in malignant tumors: An update. *World J Clin Cases* 2015; 3(5):393-404.
42. Cai J, Chen Z, Zuo X. circSMARCA5 Functions as a Diagnostic and Prognostic Biomarker for Gastric Cancer. *Dis Markers* 2019; 2019:2473652.
43. Barbagallo D, Caponnetto A, Brex D, et al. CircSMARCA5 Regulates VEGFA mRNA Splicing and Angiogenesis in Glioblastoma Multiforme Through the Binding of SRSF1. *Cancers (Basel)* 2019; 11(2).
44. Kong Z, Wan X, Zhang Y, et al. Androgen-responsive circular RNA circSMARCA5 is up-regulated and promotes cell proliferation in prostate cancer. *Biochem Biophys Res Commun* 2017; 493(3):1217-1223.
45. Zeng K, Chen X, Xu M, et al. CircHIPK3 promotes colorectal cancer growth and metastasis by sponging miR-7. *Cell Death Dis* 2018; 9(4):417.
46. Vo JN, Cieslik M, Zhang Y, et al. The Landscape of Circular RNA in Cancer. *Cell* 2019; 176(4):869-881 e13.
47. Mathsyaraja H, Catchpole J, Eastwood E, et al. Loss of MGA mediated Polycomb repression promotes tumor progression and invasiveness. *bioRxiv* 2020:2020.10.16.334714.
48. Zhang N, Nan A, Chen L, et al. Circular RNA circSATB2 promotes progression of non-small cell lung cancer cells. *Mol Cancer* 2020; 19(1):101.
49. Wu L, Hui H, Wang LJ, et al. MicroRNA-326 functions as a tumor suppressor in colorectal cancer by targeting the nin one binding protein. *Oncol Rep* 2015; 33(5):2309-18.
50. Zhang X, Zhao Y, Kong P, et al. Expression of circZNF609 is Down-Regulated in Colorectal Cancer Tissue and Promotes Apoptosis in Colorectal Cancer Cells by Upregulating p53. *Med Sci Monit* 2019; 25:5977-5985.
51. Dai J, Zhuang Y, Tang M, et al. CircRNA UBAP2 facilitates the progression of colorectal cancer by regulating miR-199a/VEGFA pathway. *Eur Rev Med Pharmacol Sci* 2020; 24(15):7963-7971.
52. Buratin A, Paganin M, Gaffo E, et al. Large-scale circular RNA deregulation in T-ALL: unlocking unique ectopic expression of molecular subtypes. *Blood Adv* 2020; 4(23):5902-5914.
53. He Y, Huang H, Jin L, et al. CircZNF609 enhances hepatocellular carcinoma cell proliferation, metastasis, and stemness by activating the Hedgehog pathway through the regulation of miR-15a-5p/15b-5p and GLI2 expressions. *Cell Death Dis* 2020; 11(5):358.
54. Li M, Li Y, Yu M. CircRNA ZNF609 Knockdown Suppresses Cell Growth via Modulating miR-188/ELF2 Axis in Nasopharyngeal Carcinoma. *Onco Targets Ther* 2020; 13:2399-2409.
55. Liu Z, Pan HM, Xin L, et al. Circ-ZNF609 promotes carcinogenesis of gastric cancer cells by inhibiting miRNA-145-5p expression. *Eur Rev Med Pharmacol Sci* 2019; 23(21):9411-9417.
56. Zhu L, Liu Y, Yang Y, et al. CircRNA ZNF609 promotes growth and metastasis of nasopharyngeal carcinoma by competing with microRNA-150-5p. *Eur Rev Med Pharmacol Sci* 2019; 23(7):2817-2826.
57. Rossi F, Legnini I, Megiorni F, et al. Circ-ZNF609 regulates G1-S progression in rhabdomyosarcoma. *Oncogene* 2019; 38(20):3843-3854.
58. Xu L, Ma Y, Zhang H, et al. HMGA2 regulates circular RNA ASPH to promote tumor growth in lung adenocarcinoma. *Cell Death Dis* 2020; 11(7):593.
59. He Q, Yan D, Dong W, et al. circRNA circFUT8 Upregulates Krupple-like Factor 10 to Inhibit the Metastasis of Bladder Cancer via Sponging miR-570-3p. *Mol Ther Oncolytics* 2020; 16:172-187.

Supplementary Data

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Supplementary Table 1. Candidate circular RNA validation by Sanger Sequencing.

Circular Region	Ensembl Gene ID	Gene	Oligonucleotide	Sequence (5'–3')	Tm (°C)	Amplicon Size (bp)
chr2:199368605-199433515	ENSG00000119042	*SATB2*	Forward	CAAACTCGGCGTGTTCTTCTC	59.9	148
			Reverse	AATGTGTCAGCAACCAAGTGC	58.3	
chr7:152309966-152315339	ENSG00000055609	*KMT2C*	Forward	ATCCAGGCGTTATTCTGAATTGT	59.9	147
			Reverse	TGCCAGATGGAAGAACCATTG	60.71	
chr12:95208843-95211268	ENSG00000180263	*FGD6*	Forward	ATTTGCCACAACAAACTTGGG	60.1	149
			Reverse	CAGCATGGAGGACGCTGAT	58.9	
chr2:207976651-207977587	ENSG00000178385	*PLEKHM3*	Forward	CTGCATAACAGCTTTGCCAGT	58.3	135
			Reverse	TGTCTTTGGAACGAGGACTCA	57.9	

Supplementary Table 2. circRNAs that were observed in 20 samples or more.

Circular Region	Ensemble Gene ID	Gene	Exons	# Samples	R *	circID
chr4:143543509-143543973	ENSG00000153147	*SMARCA5*	15-16	177	0.295	hsa_circ_0001445
chr11:33286413-33287512	ENSG00000110422	*HIPK3*	2	177	0.541	hsa_circ_0000284
chr7:100023419-100024308	ENSG00000106261	*ZKSCAN1*	2-3	177	0.539	hsa_circ_0001727
chr4:152411303-152412530	ENSG00000109670	*FBXW7*	2	177	0.437	hsa_circ_0001451
chr1:117402186-117420650	ENSG00000198162	*MAN1A2*	2-5	172	0.489	hsa_circ_0000118
chr14:99458279-99465814	ENSG00000183576	*SETD3*	6-2	170	0.352	hsa_circ_0000567
chr5:123545417-123557565	ENSG00000151292	*CSNK1G3*	2-3	166	0.52	hsa_circ_0001522
chr14:32090502-32094387	ENSG00000100852	*ARHGAP5*	3-2	162	0.64	hsa_circ_0031583
chr19:23358430-23362726	ENSG00000167232	*ZNF91*	1-4	161	0.756	
chr14:32090502-32117288	ENSG00000100852	*ARHGAP5*	3	157	0.607	hsa_circ_0031584
chr5:168488602-168494651	ENSG00000113643	*RARS*	2-5	156	0.276	hsa_circ_0001550
chr20:32366384-32369124	ENSG00000171456	*ASXL1*	2-4	152	0.415	hsa_circ_0001136
chr10:7797047-7802855	ENSG00000165629	*ATP5C1*	3-8	151	0.341	hsa_circ_0007292
chr8:51860845-51861247	ENSG00000168300	*PCMTD1*	3	150	0.431	hsa_circ_0001801
chr5:137985257-137988316	ENSG00000031003	*FAM13B*	10-8	150	0.235	hsa_circ_0001535
chr2:61522611-61533904	ENSG00000082898	*XPO1*	4-2	147	0.194	hsa_circ_0001017
chr13:32517857-32527533	ENSG00000244754	*N4BP2L2*	5-2	144	0.463	hsa_circ_0000471
chr7:155672867-155680909	ENSG00000184863	*RBM33*	3-5	142	0.121	hsa_circ_0001772
chr6:47283938-47286596	ENSG00000146072	*TNFRSF21*	3-2	141	0.548	hsa_circ_0001610
chr8:61680968-61684189	ENSG00000198363	*ASPH*	3-2	139	0.525	hsa_circ_0084615
chr6:4891713-4892380	ENSG00000153046	*CDYL*	4	139	0.524	hsa_circ_0008285
chr7:22291175-22318038	ENSG00000136237	*RAPGEF5*	1-2	128	0.474	hsa_circ_0001681
chr17:20204333-20205913	ENSG00000128487	*SPECC1*	4	122	0.678	hsa_circ_0000745
chr15:58912563-58917000	ENSG00000137776	*SLTM*	5-3	119	0.203	hsa_circ_0000605
chr15:64499293-64500167	ENSG00000180357	*ZNF609*	2-1	117	0.368	hsa_circ_0000615
chr9:33971651-33973238	ENSG00000137073	*UBAP2*	8-7	116	0.156	hsa_circ_0001851
chr12:108652272-108654411	ENSG00000110880	*CORO1C*	8-7	115	0.212	hsa_circ_0000437
chr1:117402186-117442326	ENSG00000198162	*MAN1A2*	2-6	115	0.275	hsa_circ_0000119
chr12:69800209-69801722	ENSG00000127328	*RAB3IP*	7-8	114	0.293	hsa_circ_0000419
chr15:25405461-25411972	ENSG00000114062	*UBE3A*	4-2	112	0.356	hsa_circ_0000586
chr4:55411614-55417986	ENSG00000134851	*TMEM165*	2-4	112	0.339	hsa_circ_0001414
chr6:158312051-158314269	ENSG00000130338	*TULP4*	2-1	110	0.476	
chr4:128992167-129003877	ENSG00000151466	*SCLT1*	9-6	109	0.339	hsa_circ_0001439
chr20:35714185-35725156	ENSG00000131051	*RBM39*	10-6	109	0.407	hsa_circ_0004870
chr11:130260856-130261930	ENSG00000196323	*ZBTB44*	2	109	0.317	hsa_circ_0002484
chr2:40428473-40430305	ENSG00000183023	*SLC8A1*	2	107	0.61	hsa_circ_0000994
chr14:96833467-96860736	ENSG00000100749	*VRK1*	2-11	105	0.271	hsa_circ_0000566
chr3:149846011-149921228	ENSG00000082996	*RNF13*	2-8	102	0.512	hsa_circ_0001346
chr6:18236452-18258406	ENSG00000124795	*DEK*	9-3	102	0.446	hsa_circ_0075796
chr21:36247517-36248569	ENSG00000142197	*DOPEY2*	20-21	101	0.388	hsa_circ_0001187
chr4:186706563-186709846	ENSG00000083857	*FAT1*	2	96	0.339	hsa_circ_0001461

Supplementary Tabel 2. circRNAs that were observed in 20 samples or more. (continued)

Circular Region	Ensemble Gene ID	Gene	Exons	# Samples	R *	circID
chr7:158759486-158764854	ENSG00000117868	*ESYT2*	13-9	94	0.398	hsa_circ_0001776
chr2:190659158-190673153	ENSG00000138386	*NAB1*	2-4	94	0.661	hsa_circ_0002024
chr15:41668828-41669959	ENSG00000174197	*MGA*	2	94	0.09	hsa_circ_0000591
chr1:7777160-7778170	ENSG00000049245	*VAMP3*	3-4	93	0.05	hsa_circ_0006354
chr9:33953285-33963792	ENSG00000137073	*UBAP2*	12-9	91	0.247	hsa_circ_0001847
chr2:199368605-199433515	ENSG00000119042	*SATB2*	6-3	90	0.607	hsa_circ_0003915
chr3:56592970-56594029	ENSG00000180376	*CCDC66*	8-9	90	0.295	hsa_circ_0001313
chr9:135881633-135883079	ENSG00000130559	*CAMSAP1*	3-2	90	0.197	hsa_circ_0001900
chr10:126970702-127127765	ENSG00000150760	*DOCK1*	2-27	89	0.504	hsa_circ_0020397
chr9:93471141-93498887	ENSG00000048828	*FAM120A*	2	89	0.403	hsa_circ_0001875
chr14:22909483-22911404	ENSG00000100461	*RBM23*	3-2	88	0.154	hsa_circ_0000524
chr8:141253989-141254630	ENSG00000022567	*SLC45A4*	1-2	87	0.05	hsa_circ_0001829
chr7:22308339-22318038	ENSG00000136237	*RAPGEF5*	5-2	86	0.284	
chr1:31915895-31919659	ENSG00000184007	*PTP4A2*	2-2	86	0.439	hsa_circ_0007364
chr3:158122103-158123992	ENSG00000174891	*RSRC1*	2-3	85	0.295	hsa_circ_0001355
chr1:205616478-205623892	ENSG00000158711	*ELK4*	5-2	84	0.405	hsa_circ_0000175
chr17:45475100-45475727	ENSG00000225190	*PLEKHM1*	4	84	0.383	hsa_circ_0044177
chr7:39987599-40002032	ENSG00000065883	*CDK13*	2-5	84	0.481	hsa_circ_0001699
chr21:15762891-15766142	ENSG00000155313	*USP25*	2-3	84	0.11	hsa_circ_0001178
chr4:128074460-128077963	ENSG00000138709	*LARP1B*	2-4	79	0.32	hsa_circ_0001438
chr13:60439688-60467380	ENSG00000083544	*TDRD3*	2-4	77	0.529	hsa_circ_0003441
chr17:1050050-1100736	ENSG00000159842	*ABR*	16-3	76	0.468	hsa_circ_0007919
chr4:87195324-87195691	ENSG00000145332	*KLHL8*	2	76	0.313	hsa_circ_0002538
chr1:1223244-1223969	ENSG00000078808	*SDF4*	4-3	76	0.224	hsa_circ_0000002
chr2:72718103-72733119	ENSG00000144036	*EXOC6B*	6-3	75	0.287	hsa_circ_0009043
chr9:111386377-111391825	ENSG00000136813	*KIAA0368*	31-28	75	–0.072	hsa_circ_0001882
chr8:18799295-18804899	ENSG00000156011	*PSD3*	8-5	73	0.463	hsa_circ_0004458
chr12:32598497-32611284	ENSG00000139132	*FGD4*	5-10	72	0.555	hsa_circ_0025843
chr9:110972073-110973559	ENSG00000198121	*LPAR1*	3-2	71	0.494	hsa_circ_0087960
chr18:12999421-13019207	ENSG00000101639	*CEP192*	2-9	70	0.564	hsa_circ_0000831
chr12:46229153-46243315	ENSG00000111371	*SLC38A1*	5-2	70	0.383	hsa_circ_0000396
chr4:37631385-37638505	ENSG00000181826	*RELL1*	6-4	70	0.381	hsa_circ_0001400
chr6:158282263-158314269	ENSG00000130338	*TULP4*	1-2	69	0.406	
chr5:95755396-95763621	ENSG00000164292	*RHOBTB3*	6-7	69	0.575	hsa_circ_0007444
chr3:170359699-170361430	ENSG00000136603	*SKIL*	2	67	0.543	hsa_circ_0067938
chr6:116689320-116692393	ENSG00000196911	*KPNA5*	3-5	66	0.31	
chr16:85633914-85634133	ENSG00000131149	*GSE1*	2	65	0.19	hsa_circ_0000722
chr5:73074742-73077494	ENSG00000157107	*FCHO2*	20-21	65	0.421	hsa_circ_0002490
chr2:61522611-61526522	ENSG00000082898	*XPO1*	4-3	65	0.235	hsa_circ_0001016
chr3:170136419-170149245	ENSG00000173889	*PHC3*	7-5	64	0.631	hsa_circ_0001359
chr21:29321221-29329694	ENSG00000156273	*BACH1*	2-4	63	0.404	hsa_circ_0001181

Supplementary Tabel 2. circRNAs that were observed in 20 samples or more. (continued)

Circular Region	Ensemble Gene ID	Gene	Exons	# Samples	R *	circID
chr14:65561337-65561767	ENSG00000033170	*FUT8*	3	63	0.519	hsa_circ_0003028
chr12:120154970-120155720	ENSG00000089154	*GCN1L1*	31-29	63	0.144	hsa_circ_0000448
chr12:27714780-27724187	ENSG00000061794	*MRPS35*	2-5	62	0.453	hsa_circ_0000384
chr14:21503173-21503882	ENSG00000165819	*METTL3*	2	61	–0.012	hsa_circ_0000523
chr15:89113725-89116522	ENSG00000140526	*ABHD2*	2-3	61	0.397	hsa_circ_0007099
chr20:58438945-58441084	ENSG00000124164	*VAPB*	4-5	61	0.436	hsa_circ_0001173
chr2:58221942-58232113	ENSG00000115392	*FANCL*	5-2	60	0.559	hsa_circ_0001009
chr1:23030469-23050521	ENSG00000004487	*KDM1A*	2-3	59	0.133	hsa_circ_0009061
chr18:12999421-13030609	ENSG00000101639	*CEP192*	2-11	58	0.509	
chr7:24623666-24668661	ENSG00000105926	*MPP6*	2-9	58	0.762	hsa_circ_0001686
chr1:155853276-155853807	ENSG00000116580	*GON4L*	2	58	–0.28	hsa_circ_0000139
chr8:130152736-130180881	ENSG00000153317	*ASAP1*	7-8	58	0.253	hsa_circ_0008934
chr7:129014979-129018158	ENSG00000064419	*TNPO3*	4-2	58	0.208	hsa_circ_0001741
chr9:86305192-86310018	ENSG00000083223	*ZCCHC6*	24-20	57	0.419	hsa_circ_0001869
chr4:185247294-185267156	ENSG00000109762	*SNX25*	2-5	57	0.618	hsa_circ_0004874
chr7:100812747-100813209	ENSG00000196411	*EPHB4*	12-11	55	0.342	hsa_circ_0001730
chr19:12928342-12928848	ENSG00000179115	*FARSA*	8-6	55	0.025	hsa_circ_0000896
chr2:112299849-112300030	ENSG00000188177	*ZC3H6*	2-2	55	0.556	hsa_circ_0001062
chr3:170145423-170149245	ENSG00000173889	*PHC3*	6-5	54	0.69	hsa_circ_0001360
chrX:131749306-131794467	ENSG00000213468	*FIRRE*	10-5	53	0.808	hsa_circ_0001944
chr9:37126312-37126943	ENSG00000147905	*ZCCHC7*	2	52	0.407	hsa_circ_0001860
chr3:63912588-63913226	ENSG00000163635	*ATXN7*	4	52	0.25	hsa_circ_0007761
chr1:224952670-224974154	ENSG00000185842	*DNAH14*	2-8	51	0.412	hsa_circ_0016600
chr1:41070595-41075452	ENSG00000010803	*SCMH1*	9-8	50	–0.025	hsa_circ_0000061
chr13:95757644-95763954	ENSG00000102580	*DNAJC3*	5-9	50	0.241	
chr1:155438327-155459899	ENSG00000116539	*ASH1L*	5-4	49	0.034	hsa_circ_0003247
chr4:76134175-76144474	ENSG00000138750	*NUP54*	4-2	49	0.199	hsa_circ_0070039
chr2:99169550-99171430	ENSG00000158411	*MITD1*	3-4	48	0.045	hsa_circ_0001050
chr8:140864312-140890770	ENSG00000169398	*PTK2*	5-3	48	0.031	hsa_circ_0002483
chr13:75560753-75569508	ENSG00000118939	*UCHL3*	3-6	48	0.353	hsa_circ_0000494
chr3:172247533-172251542	ENSG00000075420	*FNDC3B*	5-6	47	0.362	hsa_circ_0006156
chr10:101667886-101676437	ENSG00000107829	*FBXW4*	5-2	47	–0.051	hsa_circ_0008362
chr8:127890589-127890999	ENSG00000249859	*PVT1*	3-2	47	0.356	hsa_circ_0001821
chr15:62007308-62013993	ENSG00000129003	*VPS13C*	13-8	46	0.166	hsa_circ_0000607
chr10:110964125-110985766	ENSG00000108061	*SHOC2*	2-3	46	0.349	hsa_circ_0020028
chr16:69370483-69372356	ENSG00000132604	*TERF2*	5-4	46	–0.069	
chr3:125313308-125331239	ENSG00000163848	*ZNF148*	4-2	46	0.363	hsa_circ_0001333
chr14:39179091-39179463	ENSG00000100941	*PNN*	6-8	46	–0.02	
chr8:94664697-94665197	ENSG00000104413	*ESRP1*	7-9	46	0.291	hsa_circ_0084927
chr2:147896301-147899899	ENSG00000121989	*ACVR2A*	2-4	45	0.341	hsa_circ_0001073
chr19:8455405-8463687	ENSG00000099783	*HNRNPM*	2-5	45	–0.223	hsa_circ_0006382

Supplementary Tabel 2. circRNAs that were observed in 20 samples or more. (continued)

Circular Region	Ensemble Gene ID	Gene	Exons	# Samples	R *	circID
chr7:152309966-152315339	ENSG00000055609	*KMT2C*	6-4	45	–0.033	hsa_circ_0001769
chr17:51263274-51268905	ENSG00000011260	*UTP18*	2-4	44	0.132	hsa_circ_0002789
chr14:102040236-102040674	ENSG00000197102	*DYNC1H1*	63-64	44	–0.212	hsa_circ_0002398
chr20:33619517-33623297	ENSG00000078699	*CBFA2T2*	4-5	44	0.264	hsa_circ_0003426
chr4:73090667-73092301	ENSG00000132466	*ANKRD17*	29	44	0.204	hsa_circ_0007883
chr7:23611171-23611554	ENSG00000169193	*CCDC126*	3	43	0.49	hsa_circ_0001684
chr5:57246300-57251142	ENSG00000062194	*GPBP1*	8-11	43	0.253	hsa_circ_0072547
chr7:158788004-158799073	ENSG00000117868	*ESYT2*	6-2	43	0.551	hsa_circ_0001777
chr18:21765772-21779686	ENSG00000101752	*MIB1*	2-6	42	0.513	hsa_circ_0000835
chr5:128138597-128152806	ENSG00000064651	*SLC12A2*	8-15	42	0.598	hsa_circ_0006034
chr7:156826605-156836886	ENSG00000105983	*LMBR1*	4-2	42	0.345	hsa_circ_0005939
chr3:138570318-138571357	ENSG00000114107	*CEP70*	6-4	41	0.539	hsa_circ_0002468
chr12:120782655-120784594	ENSG00000157837	*SPPL3*	6-4	41	–0.013	hsa_circ_0003472
chr12:123586747-123590450	ENSG00000086598	*TMED2*	2-3	41	0.265	hsa_circ_0000458
chr2:202464809-202467690	ENSG00000204217	*BMPR2*	2-3	41	0.183	hsa_circ_0003218
chr2:8908621-8958643	ENSG00000143797	*MBOAT2*	4-2	40	0.71	hsa_circ_0000972
chr2:88782734-88792495	ENSG00000230006	*ANKRD36BP2*	4-12	40	0.465	
chr4:128074460-128082306	ENSG00000138709	*LARP1B*	2-5	40	0.493	hsa_circ_0007619
chr5:145817894-145826201	ENSG00000186314	*PRELID2*	5-2	39	0.562	hsa_circ_0006528
chr1:117402186-117405646	ENSG00000198162	*MAN1A2*	2-3	39	0.444	hsa_circ_0000116
chr12:95208843-95211268	ENSG00000180263	*FGD6*	2	38	0.39	
chr2:233388257-233390484	ENSG00000077044	*DGKD*	2-3	38	–0.242	hsa_circ_0001112
chr8:60741259-60743098	ENSG00000171316	*CHD7*	2	38	0.253	hsa_circ_0084582
chr19:47264603-47264947	ENSG00000105321	*CCDC9*	6-7	38	0.375	hsa_circ_0000944
chr11:77624963-77625819	ENSG00000074201	*CLNS1A*	4-3	38	0.451	hsa_circ_0000343
chr12:100282943-100298176	ENSG00000136021	*SCYL2*	2-4	38	0.178	hsa_circ_0006258
chr15:90439332-90443479	ENSG00000140575	*IQGAP1*	6-9	37	0.532	hsa_circ_0000651
chr7:66127704-66134375	ENSG00000249319	*AC068533.7*	8-10	37		hsa_circ_0004604
chr4:177353308-177360678	ENSG00000109674	*NEIL3*	8-9	37	0.307	hsa_circ_0001460
chr10:68959806-68960250	ENSG00000165732	*DDX21*	2	37	0.266	hsa_circ_0008865
chr6:13579451-13584226	ENSG00000124523	*SIRT5*	2-3	36	0.098	hsa_circ_0007218
chr1:59339958-59378838	ENSG00000172456	*FGGY*	3-5	36	0.218	hsa_circ_0006633
chr19:5604583-5604937	ENSG00000130254	*SAFB2*	11-10	36	0.017	hsa_circ_0000880
chr2:189791790-189818181	ENSG00000064933	*PMS1*	2-5	36	0.133	hsa_circ_0001083
chr8:37765526-37766356	ENSG00000147471	*PROSC*	2-4	36	0.184	hsa_circ_0001788
chr2:134253095-134254645	ENSG00000152127	*MGAT5*	2-3	35	0.228	hsa_circ_0001068
chr14:21230319-21234230	ENSG00000092199	*HNRNPC*	4-2	35	0.165	hsa_circ_0003643
chr3:146121112-146124230	ENSG00000152952	*PLOD2*	3-2	35	0.409	
chr11:77619606-77625819	ENSG00000074201	*CLNS1A*	1-3	35	0.326	hsa_circ_0023694
chr5:128131067-128141982	ENSG00000064651	*SLC12A2*	5-10	35	0.242	hsa_circ_0073762
chr3:196391813-196403020	ENSG00000163960	*UBXN7*	5-3	35	0.214	hsa_circ_0001380
chr1:30992390-30995221	ENSG00000134644	*PUM1*	7-6	35	0.199	hsa_circ_0000043

Supplementary Tabel 2. circRNAs that were observed in 20 samples or more. (continued)

Circular Region	Ensemble Gene ID	Gene	Exons	# Samples	R *	circID
chr15:32526813-32533369	ENSG00000223509	*RP11-632K20.7*	1-2	34	0.579	
chr1:805799-810171	ENSG00000230092	*RP11-206L10.8*	4-2	34	0.633	hsa_circ_0002333
chr6:7176655-7189323	ENSG00000124782	*RREB1*	2-6	34	–0.203	hsa_circ_0001573
chr4:102304317-102315831	ENSG00000138821	*SLC39A8*	6-3	33	0.451	hsa_circ_0002782
chr12:28225795-28259443	ENSG00000123106	*CCDC91*	2-4	33	0.451	hsa_circ_0000386
chr10:126970702-127257430	ENSG00000150760	*DOCK1*	2-29	33	0.514	hsa_circ_0020399
chr15:80120328-80122801	ENSG00000086666	*ZFAND6*	3-5	33	0.01	hsa_circ_0000643
chr5:65988635-65994865	ENSG00000112851	*ERBB2IP*	2-4	33	0.407	hsa_circ_0001492
chr1:35358925-35361790	ENSG00000146463	*ZMYM4*	3-5	33	0.172	hsa_circ_0011536
chr8:108449823-108455931	ENSG00000104412	*EMC2*	3-5	33	0.175	
chr2:214767482-214781510	ENSG00000138376	*BARD1*	6-4	33	0.548	hsa_circ_0001098
chr2:207976651-207977587	ENSG00000178385	*PLEKHM3*	3	33	–0.168	hsa_circ_0001095
chr16:47497399-47515602	ENSG00000102893	*PHKB*	3-7	32	0.118	hsa_circ_0000698
chr11:85996826-86031612	ENSG00000073921	*PICALM*	12-2	32	0.18	hsa_circ_0023923
chr20:35716740-35725156	ENSG00000131051	*RBM39*	9-6	32	0.479	hsa_circ_0001147
chr10:84438512-84477665	ENSG00000107771	*CCSER2*	6-9	32	0.532	hsa_circ_0018998
chr6:138943513-138944623	ENSG00000135597	*REPS1*	7-5	32	0.45	hsa_circ_0004368
chr4:3086939-3107424	ENSG00000197386	*HTT*	2-5	32	–0.057	hsa_circ_0001392
chr4:48369849-48383785	ENSG00000109171	*SLAIN2*	2-6	31	0.413	
chr11:73707420-73718719	ENSG00000175582	*RAB6A*	6-4	31	0.373	hsa_circ_0000339
chr11:61366045-61367999	ENSG00000149483	*TMEM138*	3-4	31	0.098	hsa_circ_0002058
chr3:138570318-138572933	ENSG00000114107	*CEP70*	6-3	30	0.382	hsa_circ_0004524
chr1:45640210-45642500	ENSG00000159592	*GPBP1L1*	7-6	30	0.149	hsa_circ_0008774
chr10:31908172-31910564	ENSG00000165322	*ARHGAP12*	1-2	30	0.536	hsa_circ_0000231
chr2:106158058-106166084	ENSG00000115652	*UXS1*	5-2	29	0.004	hsa_circ_0001060
chr2:201145378-201149836	ENSG00000003402	*CFLAR*	6-8	29	0.093	hsa_circ_0001092
chr5:145796442-145826201	ENSG00000186314	*PRELID2*	1-2	29	0.545	hsa_circ_0008647
chr3:71041328-71053774	ENSG00000114861	*FOXP1*	11-8	29	0.845	hsa_circ_0008234
chr11:32927157-32935436	ENSG00000060749	*QSER1*	2-4	29	0.38	hsa_circ_0021570
chr6:158580940-158589783	ENSG00000146433	*TMEM181*	3-6	29	0.348	hsa_circ_0001661
chr1:26942660-26943066	ENSG00000090273	*NUDC*	7-6	29	0.159	hsa_circ_0005087
chr11:18291442-18292977	ENSG00000110756	*HPS5*	15-14	29	0.152	hsa_circ_0000280
chr7:22266964-22318038	ENSG00000136237	*RAPGEF5*	2	29	0.337	hsa_circ_0079557
chr8:37877109-37877552	ENSG00000156675	*RAB11FIP1*	2	28	0.229	hsa_circ_0001789
chr1:155438327-155439069	ENSG00000116539	*ASH1L*	5	28	0.091	hsa_circ_0000137
chr20:35729312-35732136	ENSG00000131051	*RBM39*	5-3	28	0.341	hsa_circ_0008817
chr9:33960826-33973238	ENSG00000137073	*UBAP2*	10-7	28	0.435	hsa_circ_0001850
chr8:61618978-61653661	ENSG00000198363	*ASPH*	14-4	28	0.429	hsa_circ_0084606
chr15:41696075-41699160	ENSG00000174197	*MGA*	2-5	28	–0.244	hsa_circ_0000592
chr9:37424845-37426655	ENSG00000137106	*GRHPR*	2-4	27	0.26	hsa_circ_0001861

Supplementary Tabel 2. circRNAs that were observed in 20 samples or more. (continued)

Circular Region	Ensemble Gene ID	Gene	Exons	# Samples	R *	circID
chr5:154033791-154034968	ENSG00000055147	*FAM114A2*	4-2	27	0.45	hsa_circ_0001546
chr10:100923975-100926020	ENSG00000119906	*FAM178A*	5-6	27	0.378	hsa_circ_0006654
chr8:102360071-102361627	ENSG00000104517	*UBR5*	5-2	26	0.07	hsa_circ_0001819
chr6:13632370-13644730	ENSG00000010017	*RANBP9*	12-6	26	0.371	hsa_circ_0001577
chr6:30650994-30651467	ENSG00000204564	*C6orf136*	3-4	26	0.102	hsa_circ_0006109
chr1:20757166-20773611	ENSG00000127483	*HP1BP3*	9-5	26	–0.079	hsa_circ_0000024
chr14:63998914-64022864	ENSG00000054654	*SYNE2*	27-38	26	0.099	
chr9:125337018-125337592	ENSG00000165219	*GAPVD1*	14-15	26	0.152	hsa_circ_0003270
chr10:104008177-104018909	ENSG00000065613	*SLK*	12-14	26	0.366	hsa_circ_0000259
chrX:85303406-85308217	ENSG00000124429	*POF1B*	15-10	26	0.321	hsa_circ_0091187
chr1:70292388-70315567	ENSG00000118454	*ANKRD13C*	9-4	26	0.355	hsa_circ_0000085
chr4:105424196-105456746	ENSG00000138777	*PPA2*	7-2	26	0.431	hsa_circ_0001434
chr19:29985223-29986418	ENSG00000105176	*URI1*	3-4	25	0.116	hsa_circ_0000921
chr16:18841565-18845652	ENSG00000157106	*SMG1*	41-39	25	0.152	hsa_circ_0006434
chr7:131375424-131399434	ENSG00000128585	*MKLN1*	3-7	25	0.531	hsa_circ_0001746
chr5:36953618-36976403	ENSG00000164190	*NIPBL*	2-9	25	0.554	hsa_circ_0001472
chr9:96522506-96565484	ENSG00000081377	*CDC14B*	12-2	25	0.545	hsa_circ_0087641
chr7:92294889-92327901	ENSG00000001629	*ANKIB1*	2-5	25	0.332	
chr10:124681607-124682380	ENSG00000258539	*RP11-12J10.3*	9	25	–0.252	hsa_circ_0000267
chr2:230442937-230450256	ENSG00000067066	*SP100*	3-8	24	0.023	hsa_circ_0003922
chr5:176943335-176958155	ENSG00000087206	*UIMC1*	10-7	24	–0.07	hsa_circ_0001558
chr9:93471141-93476339	ENSG00000048828	*FAM120A*	2-3	24	0.419	hsa_circ_0008193
chr8:37870420-37877552	ENSG00000156675	*RAB11FIP1*	4-2	24	0.533	hsa_circ_0005630
chr10:84371014-84373816	ENSG00000107771	*CCSER2*	2-3	24	0.627	hsa_circ_0018992
chr20:41533050-41551361	ENSG00000124177	*CHD6*	3-2	24	0.218	hsa_circ_0001159
chr2:238182065-238185288	ENSG00000132323	*ILKAP*	9-6	24	0.278	hsa_circ_0001116
chr7:158869855-158876692	ENSG00000126870	*WDR60*	2-4	24	0.29	hsa_circ_0001778
chr1:112653598-112659780	ENSG00000116489	*CAPZA1*	4-7	24	0.144	hsa_circ_0000109
chr16:88027483-88038012	ENSG00000172530	*BANP*	7-10	23	0.052	hsa_circ_0040823
chr19:5047476-5082505	ENSG00000127663	*KDM4B*	6-9	23	0.055	hsa_circ_0002926
chr1:224952670-225007545	ENSG00000185842	*DNAH14*	2-8	23	0.637	hsa_circ_0016601
chr7:27629371-27649634	ENSG00000106049	*HIBADH*	4-2	23	0.194	hsa_circ_0006773
chr2:199380364-199433515	ENSG00000119042	*SATB2*	5-3	23	0.322	hsa_circ_0002867
chr19:40583398-40583718	ENSG00000160410	*SHKBP1*	11-12	23	–0.259	hsa_circ_0000936
chr1:29154696-29154911	ENSG00000116350	*SRSF4*	4	23	–0.062	hsa_circ_0006602
chr8:17743604-17755962	ENSG00000129422	*MTUS1*	2	23	0.424	hsa_circ_0083444
chr8:18765449-18804899	ENSG00000156011	*PSD3*	9-5	23	0.195	hsa_circ_0002111
chr1:20770930-20773611	ENSG00000127483	*HP1BP3*	6-5	23	–0.006	hsa_circ_0005782
chr1:8655973-8656442	ENSG00000142599	*RERE*	3	23	–0.428	
chr8:47396376-47407962	ENSG00000164808	*SPIDR*	6-7	22	0.017	hsa_circ_0001798
chr3:134188837-134195183	ENSG00000163785	*RYK*	9-7	22	0.102	hsa_circ_0005768

Supplementary Tabel 2. circRNAs that were observed in 20 samples or more. (continued)

Circular Region	Ensemble Gene ID	Gene	Exons	# Samples	R *	circID
chr20:35721740-35725156	ENSG00000131051	*RBM39*	8-6	22	0.339	hsa_circ_0001148
chr4:90308244-90313048	ENSG00000184305	*CCSER1*	2-3	22	0.551	
chr14:39276934-39279538	ENSG00000258941	*RP11-407N17.3*	6-8	22		hsa_circ_0000530
chr18:9524594-9525852	ENSG00000017797	*RALBP1*	5-6	22	0.175	hsa_circ_0005158
chr17:67945409-67975959	ENSG00000171634	*BPTF*	22-27	22	0.636	hsa_circ_0000799
chr10:5794885-5800706	ENSG00000057608	*GDI2*	4-2	22	–0.015	hsa_circ_0002665
chr10:89751346-89762836	ENSG00000138182	*KIF20B*	24-29	22	0.563	hsa_circ_0019079
chr16:3850297-3851010	ENSG00000005339	*CREBBP*	2	21	–0.075	hsa_circ_0007637
chr7:131387120-131399434	ENSG00000128585	*MKLN1*	4-7	21	0.018	hsa_circ_0001747
chr6:110887505-110890357	ENSG00000123505	*AMD1*	2-4	21	0.277	hsa_circ_0005954
chr22:20933779-20934245	ENSG00000099942	*CRKL*	2	21	0.192	hsa_circ_0001206
chr8:98706467-98707312	ENSG00000104375	*STK3*	6-5	21	0.016	hsa_circ_0004592
chr20:51674153-51690820	ENSG00000054793	*ATP9A*	6-8	21	0.438	hsa_circ_0004770
chr3:56660731-56673726	ENSG00000163946	*FAM208A*	4-2	21	0.272	hsa_circ_0001315
chr19:34430576-34438767	ENSG00000126261	*UBA2*	3	21	0.367	hsa_circ_0006987
chr11:85996826-86003452	ENSG00000073921	*PICALM*	12-9	21	–0.143	hsa_circ_0023919
chr5:180261684-180280609	ENSG00000050748	*MAPK9*	5-2	21	0.463	hsa_circ_0001566
chr3:67495798-67508904	ENSG00000172340	*SUCLG2*	9-7	21	0.113	hsa_circ_0004276
chr4:87046166-87047595	ENSG00000172493	*AFF1*	3-4	21	–0.145	hsa_circ_0001423
chr17:59353215-59353527	ENSG00000175155	*YPEL2*	2	20	0.048	hsa_circ_0005600
chr13:112516440-112527485	ENSG00000126216	*TUBGCP3*	17-12	20	0.215	hsa_circ_0000504
chr4:51863437-51891852	ENSG00000109184	*DCUN1D4*	2-6	20	0.135	hsa_circ_0007646
chr5:16779545-16783470	ENSG00000145555	*MYO10*	9-5	20	0.2	
chr9:83678441-83686156	ENSG00000135018	*UBQLN1*	5-2	20	0.465	hsa_circ_0087357
chr8:70213903-70216765	ENSG00000140396	*NCOA2*	4-3	20	0.516	
chr13:75621763-75727099	ENSG00000261553	*RP11-29G8.3*	8-10	20	0.314	
chr2:88801099-88804881	ENSG00000230006	*ANKRD36BP2*	13	20	0.595	
chr16:69695136-69695380	ENSG00000102908	*NFAT5*	15	20	0.347	hsa_circ_0006845
chr7:139715932-139717016	ENSG00000064393	*HIPK2*	2	20	0.691	hsa_circ_0001756
chr7:43639449-43640650	ENSG00000106603	*COA1*	6-5	20	0.403	hsa_circ_0001700
chr10:34269657-34284246	ENSG00000148498	*PARD3*	22-21	20	0.345	hsa_circ_0018168

* R indicates the Pearson correlation between the number of circRNA reads and mRNA reads for that gene.

Chapter 8

A panel of DNA methylation markers for the classification of consensus molecular subtype 2 and 3 in patients with colorectal cancer

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Under Revision

ABSTRACT

Introduction

Consensus molecular subtypes (CMSs) can guide precision treatment of colorectal cancer (CRC). We aim to identify methylation markers to distinguish between CMS2 and 3 in patients with CRC, for which an easy test is currently lacking.

Methods

Fresh-frozen tumor tissue of 239 patients with stage I-III CRC was included. Methylation profiles were obtained using the Infinium HumanMethylation450 BeadChip. We performed adaptive group-regularised logistic ridge-regression with post-hoc group-weighted elastic net marker selection to build prediction models for classification of CMS2 and CMS3. TCGA data were used for validation.

Results

Group-regularisation of the probes was done based on their location either relative to a CpG island or relative to a gene present in the CMS classifier resulting in two different prediction models and subsequently different marker panels. For both panels, even when using only 5 markers, accuracies were >90% in our cohort as well as in the TCGA validation set.

Conclusion

Our methylation marker panel accurately distinguishes between CMS2 and 3. This enables development of a targeted assay to provide a robust, clinically relevant, classification tool for CRC patients.

INTRODUCTION

The Consensus Molecular Subtypes (CMSs) classification is currently considered to be the most robust molecular stratification in colorectal cancer (CRC) with significant differences in prognosis [1]. Besides the prognostic value, literature provides some support for a predictive value of CMS for response to systemic treatment [2]. The FOxTROT study (NCT00647530) and currently ongoing CONNECTION-II trial (NTR NL8177) are expected to determine the true predictive value of CMS for response to chemotherapy. However, in general practical and affordable tests to determine CMS will greatly aid in establishing the clinical value of these molecular subtypes as these will enable routine determination of CMS in ongoing CRC research. The gold-standard classification strategy relies on genome-wide RNA expression data from sufficient quantities of fresh-frozen bulk tumor, which hampers widespread implementation. In addition, different methods can be used to classify CMS on RNA data which inherently causes differences in CMS calling per method. These classification methods include a Markov cluster algorithm (MCL), which is the algorithm applied by Guinney et al., a Random Forest Classifier (RF, based on MCL calls) and a classifier by similarity to centroid approach (Single Sample Predictor, SSP) which calls each sample independent from other samples. An affordable, robust, and practical classification assay is needed to enable both retrospective and prospective investigation of the predictive value of the CMS classification and advance its use in clinical practice. For CMS1, MSI can be used a surrogate marker given the high incidence of MSI in CMS1 tumors and the low incidence of MSI in CMS2-4 [3]. Sufficient evidence from both observational studies and randomized clinical trials is available to justify that MSI tumors represent a separate entity requiring a different treatment strategy, irrespective of their CMS classification [4, 5]. MSI testing can be done very robustly and is included in the international clinical guidelines [6]. For CMS4, an immunohistochemistry-based classifier and an RT-qPCR test have been described and validated [7, 8]. However, a more practical test to distinguish between CMS2 and 3 remains to be identified. Given the low specificity of the original CMS classification algorithm on archival formalin-fixed paraffin embedded (FFPE) tissue specimens for CMS3 and the distinct epigenomic profile in CMS3 [1], we hypothesized that DNA methylation may provide stable and useful markers to discriminate between CMS2 and CMS3. CMS3 tumors exhibit low somatic copy-number alterations (SCNAs), are hypermutated in 30% of the samples and have a low number of CpG island methylator phenotype (CIMP) cases with intermediate levels of gene hypermethylation.

Epigenetic gene silencing is one causative factor of CRC development, with DNA methylation as major driving force. Aberrant methylation in cancer is generally characterized by a diffuse DNA hypomethylation and focal hypermethylation in CpG-rich regions known as CpG islands and their surrounding shores and shelves [9, 10]. CIMP is regarded as a distinct CRC subgroup, which largely overlaps with MSI [11]. Studies suggested that the presence of CIMP plays a role

in treatment effect of chemotherapy in patients with stage II/III colon cancer [12, 13]. Furthermore, several DNA methylation biomarkers exhibit high sensitivity and specificity both in detection and prognosis of CRC [14-17]. DNA methylation markers are attractive for daily practice due to their stability, and the feasibility to detect these markers in minimally-invasive bodily fluids, stool, as well as FFPE tissue. The aim of this study was to complement currently available CMS classification tools by the identification of a panel of DNA methylation markers to distinguish CMS2 from CMS3 in patients with colorectal cancer.

METHODS

Cohort description

In the MATCH study, a multi-centred observational cohort study, fresh-frozen tumor tissue was collected from stage I-III colon cancer patients who underwent surgery between 2007 and 2014 in seven hospitals in the Rotterdam region, the Netherlands. Inclusion criteria and additional clinical characteristics of the MATCH study have been described previously [18]. For 239 patients of these patients, matched RNA expression profiles and DNA methylation profiles were generated as described below.

RNA expression profiling and CMS classification

RNA sequencing, data processing, annotation, and normalisation of these samples has been described previously [18, 19]. CMS classification was performed on the resulting RNA-seq data using the single-sample prediction parameter from the "CMSclassifier" package (https://github.com/Sage-Bionetworks/CMSclassifier). Data are available from the European Genome Phenome Archive under accession number EGAS00001002197.

DNA methylation profiling

Genomic DNA was isolated from 30 µm frozen tissue sections using the Nucleospin Tissue kit according to the manufacturer's instructions. Aforementioned RNA sequencing was performed on the same tissue section. Methylation profiles were generated from 750 ng DNA using the Infinium HumanMethylation450 BeadChip (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. This platform interrogates over 450,000 methylation sites, covering 99% of all RefSeq genes. Probes have been annotated by Illumina with respect to their position relative to gene regions (within 1500 base pairs (bp) from Transcription Start Site (TSS) (TSS1500), within 200 bp from TSS (TSS200), 5' untranslated region (5'UTR), 1st exon, gene body, 3'UTR, or intergenic region as well as relative to CpG islands (northern shelf (N-shelf),

northern shore (N-shore), CpG island, southern shore (S-shore), southern shelf (S-shelf), or open sea. Data are available from GSE164811.

Infinium HumanMethylation450 data pre-processing

Raw data was processed and normalised using the Chip Analysis Methylation Pipeline for Illumina HumanMethylation450 and EPIC (ChAMP) package in R [20, 21]. This package contains functions for filtering low-quality probes, adjustment for Infinium I and Infinium II probe design, batch effect correction, and data normalisation. In short, bad quality probes (detection p value >0.01), probes containing SNPs, probes mapping to multiple locations, and probes mapping to chromosomes X and Y were removed, resulting in 429705 probes for further analysis. Data were normalised used Beta-Mixture Quantile (BMIQ) normalisation to correct for bias between type I and type II probe chemistry and potential batch effects were removed using Combat. The returned Beta values per probe represent the percentage of methylation for that particular CpG dinucleotide.

Validation data set from TCGA

To validate the analysis results in the MATCH cohort we used data from The Cancer Genome Atlas TCGA). Matched RNAseq and Illumina HumanMethylation450 methylation data were available for 274 colorectal carcinomas. For CMS classification of these samples we again employed the single-sample prediction parameter from the "CMSclassifier" package (https://github.com/Sage-Bionetworks/CMSclassifier) to make calls between both cohorts comparable. Resulting single sample calls were also compared to the Markov Cluster model based calls originally reported in the paper by Guinney et al. to investigate the effect of using different CMS calling methods [1].

Data analysis

From the MATCH methylation dataset we first selected highly variable probes by filtering for probes with a standard deviation of at least 0.15 (Beta values) over all samples, which resulted in 52988 probes (12.3% of all probes in dataset). These probes were matched with the TCGA dataset which contained data for 45721 of these 52988 highly variable probes. All subsequent analyses were performed with these 45721 probes.

Methylation level comparisons

To compare overall methylation levels in CMS2 and CMS3 samples we calculated the median Beta value per sample over all 45721 probes as well as separately for probes located in 1) CpG islands (19873 probes), 2) shores (11111 probes: containing both north and south shores),

3) shelves (2167 probes: containing both north and south shelves, and 4) open sea (12570 probes). The obtained median methylation values were compared between CMS2 and CMS3 samples using the Wilcoxon rank sum test in the MATCH and TCGA dataset separately.

Group-regularised ridge regression analysis (grridge)

We performed adaptive group-regularised logistic ridge regression and post-hoc group-weighted elastic net feature selection as described before [22, 23]. Two types of auxiliary data were separately provided to the model for group-regularisation of the included probes: 1) CpG co-data: probe location relative to CpG island (i.e. within a CpG island (CGI), shore (northern and southern combined), shelf (northern and southern combined), or open sea) and 2) CMSori co-data: whether the CpG detected by the respective probe was associated with a gene included in the original single sample CMS Classifier (true for 1637 probes). A regression model was built with the MATCH cohort data using both types of auxiliary data and 15, 10 and 5 markers were selected by post-hoc group-weighted elastic net feature selection[23]. Performance of the model was first evaluated by 10-fold cross validation in the MATCH cohort. Predicted probabilities for the sample being CMS3 were calculated using the different models. Then, performance of the models was visualised by receiver-operator curves (ROC) and quantified by AUC. The Youden's index was calculated to determine the optimal probability cut off for the 15-, 10- and 5-marker panels based on the CpG co-data and, separately, also for the 15-, 10-, and 5-marker panels based on the CMSori co-data. Subsequently, the fixed models were applied to the TCGA cohort to validate their performance in an independent dataset. The Youden's index as determined in the MATCH dataset was used as cut-off to determine the sensitivity and specificity of the fixed models in the TCGA dataset.

Correlation analysis between DNA methylation and RNA expression

Out of the 45721 methylation probes used for predictive modeling, 24904 were located close to a gene's transcription site (TSS; up to 1500 base pairs (bp) upstream) or within a gene (either in the 5' untranslated region (UTR), the gene body or the 3'UTR). For these probes we evaluated whether the methylation level we observed in CMS2 and CMS3 samples of the MATCH cohort was associated with RNA expression of the respective gene in the same samples. Spearman correlations were calculated for every probe that was matched to a gene and a false discovery rate (FDR) correction was applied to account for multiple testing.

Multiclass classification

Samples (CMS1-4) from the MATCH and TCGA cohorts were combined and a single split was done to obtain a training (n=283) and test set (n=141). Training and test sets were balanced with respect to CMS class distribution and original cohort. We performed multi-class classification by the sparse group lasso for multinomial response, using R-package "msgl" [24] and

validated the obtained model from the training set in the test set. To obtain a more balanced representation of the four classes we double-weighted the CMS4 samples.

RESULTS

Cohort description

Matched RNAseq and Infinium 450K methylation profiles were available for 239 colon cancer patients in the MATCH cohort and 274 colorectal cancer patients in the TCGA cohort. Clinical characteristics of both cohorts are shown in Table 1. Differences in pT-stage ($p<0.001$), pN-stage ($p<0.001$), tumor stage ($p<0001$), tumor location ($p=0.023$) and CMS-classification ($p=0.001$) were seen between the two cohorts. CMS class was determined on the RNAseq data using the single sample predictor, which is independent from other samples. For the TCGA cohort, obtained CMS calls with the single sample predictor were compared to the original calls from the Markov cluster algorithm [1]. We observed a significant moderate agreement in the CMS calls obtained by the two methods (Cohen's kappa of 0.51, p=6.92E-63). However, as shown in Table 2, samples particularly shifted from CMS3 and CMS4 in the Markov cluster algorithm to NA in the Single Sample predictor and from NA in the Markov cluster algorithm to CMS2 in the single sample predictor. To ensure that CMS calling was comparable between the MATCH and TCGA cohort we therefore used the Single Sample Predictor calls for both cohorts. Then, CMS2 and CMS3 were selected from the MATCH cohort (124 CMS2 and 22 CMS3) and TCGA cohort (118 CMS2 and 22 CMS3). Within the MATCH cohort, tumor differentiation grade was significantly different between CMS2 and CMS3 and in the TCGA cohort, tumor location was significantly different between the two classes (Suppl Table 1). Principal component analysis was performed and did not show a strong separation between MATCH and TCGA samples, indicating no obvious bias was introduced by the use of the 2 different cohorts (Figure 1 & Suppl Figure 1).

Table 1. Clinical and histopathological characteristics of all patients

	MATCH		TCGA		
	N = 239	**%**	**N = 274**	**(%)**	**p-value**
Gender					0.147
Male	126	(52.7)	146	(53.3)	
Female	113	(47.3)	126	(46)	
Missing			2	(0.7)	
Age (median, IQR)	68 (61-74)		66 (55-76)		0.674
BMI (median, IQR)	26 (23.5 - 28.7)				
Tumor stage					<0.001
I	62	(25.9)	44	(16.1)	
II	108	(45.2)	105	(38.3)	
III	69	(28.9)	77	(28.1)	
IV	0	0	36	(13.1)	
Missing			12	(4.4)	
pT-stage					<0.001
Tis	0	0	1	(0.4)	
1	0	0	7	(2.6)	
2	70	(29.3)	41	(15)	
3	164	(68.6)	186	(67.9)	
4	5	(2.1)	37	(13.4)	
Missing			2	(0.7)	
pN-stage					<0.001
0	171	(71.6)	160	(58.4)	
1	44	(18.4)	67	(24.5)	
2	24	(10)	45	(16.4)	
Missing			2	(0.7)	
Tumor differentiation					
Good	22	(9.2)			
Moderate	192	(80.3)			
Poor	19	(8)			
Unknown/Other	6	(2.5)			
Tumor location					0.029
Right	126	(52.7)	162	(59.1)	
Left	113	(47.3)	95	(34.7)	
Missing			17	(6.2)	
Rectum/Colon					
Colon	239	(100)	271	(98.9)	
Rectum	0	0	1	(0.4)	
Missing			2	(0.7)	
Adjuvant therapy					
No	172	(72)			
Yes	67	(28)			

Table 1. Clinical and histopathological characteristics of all patients (continued)

	MATCH		TCGA		
	N = 239	**%**	**N = 274**	**(%)**	**p-value**
CMS					0.001
1	50	(20.9)	45	(16.4)	
2	124	(51.9)	118	(43)	
3	22	(9.2)	22	(8)	
4	8	(3.3)	35	(13.9)	
NA	35	(14.6)	54	(19.7)	
Microsatellite status					
MSS	180	(75.3)			
MSI	53	(22.2)			
Missing	6	(2.5)			

Table 2. CMS calls Markov Cluster Algorithm vs Single Sample Predictor in TCGA dataset

		CMS Single Sample Predictor					
		CMS 1	**CMS 2**	**CMS 3**	**CMS 4**	**NA**	**Total**
Markov cluster algorithm	**CMS1**	31	0	0	0	3	34
	CMS2	0	68	0	0	1	69
	CMS3	1	4	14	0	13	32
	CMS4	1	8	0	34	14	57
	NA	12	38	8	1	23	82
	Total	45	118	22	35	54	274

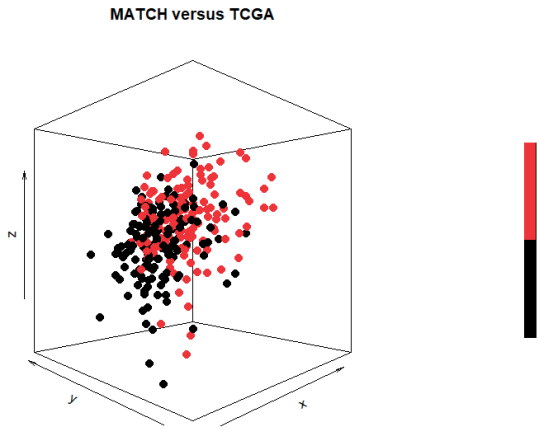


Figure 1. Principal Component Analysis (PCA) of DNA methylation profiles from all CMS2 and CMS3 samples present in the MATCH and TCGA cohorts

Principal components were calculated for DNA methylation profiles of 286 colorectal cancer tissues (146 from MATCH cohort (black) and 140 from TCGA cohort (red). PC1, PC2, and PC3 are shown on the x-, y, and z-axis respectively, where each dot represents 1 sample. Samples are colored based on their cohort of origin (MATCH in black and TCGA in red).

Comparing CMS2 and 3 DNA methylation profiles

Principal component analysis of both datasets combined showed that CMS2 and CMS3 samples are partly separated based on overall methylation profiles (Figure 2 & Suppl Figure 2). Overall, we observed a significantly higher median methylation level for our 45721 most variable probes in CMS3 compared to CMS2 (Figure 3A; Mann-Whitney U test p = 0.012 and 0.004 for MATCH and TCGA, respectively), which is in line with the observations by Guinney et al. Interestingly, when we divided probes based on their position relative to CpG islands, a difference between CMS2 and CMS3 was found for those probes located within CpG islands (Figure 3B; Mann-Whitney U test p = 2.057E-5 and 1.750E-4 for MATCH and TCGA, respectively) or their shores (Figure 3C; Mann-Whitney U test p = 0.005 and 0.002 for MATCH and TCGA, respectively), but not for probes located in shelves (Figure 3D; Mann-Whitney U test p = 0.031 and p = 0.523 for MATCH and TCGA, respectively) or open sea (Figure 3E; Mann-Whitney U test p = 0.104 and 0.964 for MATCH and TCGA, respectively).

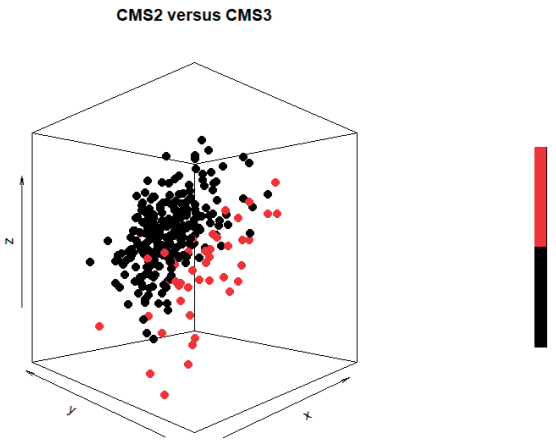


Figure 2. Principal Component Analysis (PCA) of DNA methylation profiles from all CMS2 and CMS3 samples present in the MATCH and TCGA cohorts.
Principal components were calculated for DNA methylation profiles of 286 colorectal cancer tissues (242 CMS2 samples (black) and 44 CMS3 samples). PC1, PC2, and PC3 are shown on the x-, y, and z-axis respectively, where each dot represents 1 sample. Samples are coloured based on CMS classification (CMS2 in black and CMS3).

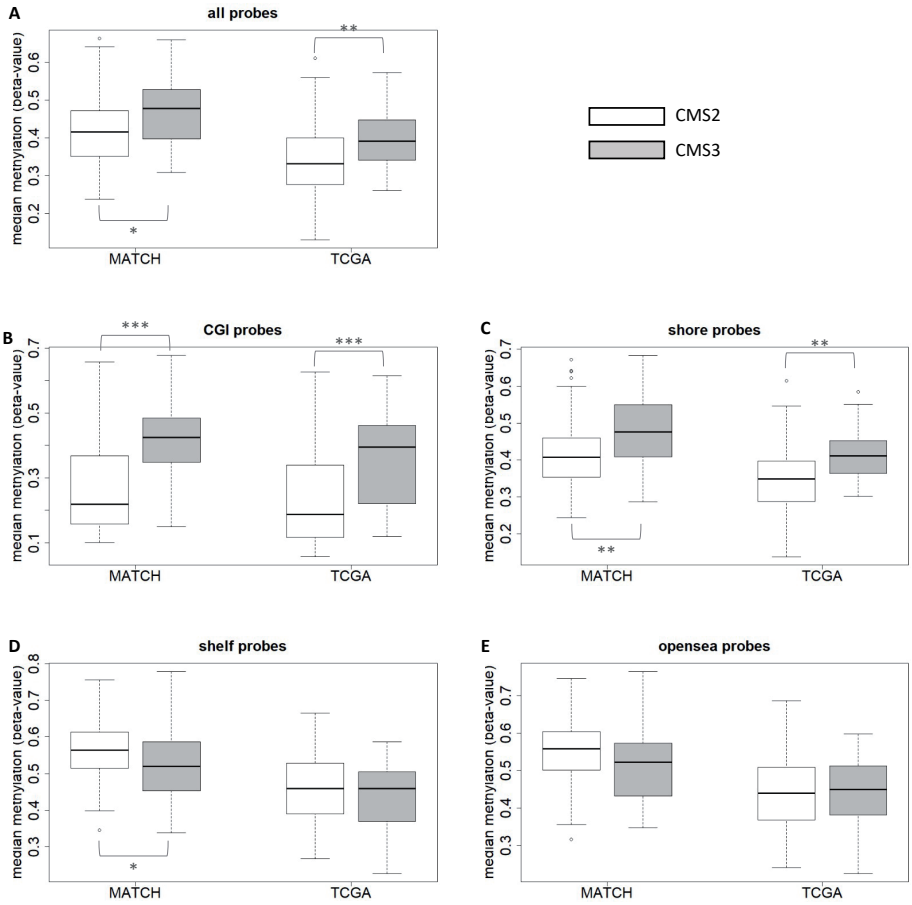


Figure 3. Box plots showing the median methylation levels observed in CMS2 and CMS3 samples where probes are grouped based on their location relative to a CpG island (CGI).
Median methylation levels are shown in CMS2 (white) and CMS3 (grey) samples from the MATCH (left) and TCGA (right) cohorts in A. for all probes included (n=45721), in B. for probes located in CpG islands (CGI; n=19837), in C. for probes located in CGI shores (n=11111), in D. for probes located in CGI shelves (n=2167), and in E. for probes located in the open sea (n=12570). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Mann-Whitney U test)

Building and validating a prediction model for CMS2 and CMS3 classification

Next, we used the MATCH dataset to build a prediction model for the classification of CMS2 and CMS3, using group-regularised logistic ridge regression (grridge) and group-weighted post-hoc feature selection [22, 23]. As shown in Figure 4A, the ordinary ridge algorithm already performed well in the classification of CMS2 versus CMS3. Group-regularisation of the probes,

based on either their relative location to a CpG island (CpG co-data panel) or their location relative to genes in the original CMS classifier (CMSori co-data panel), improved the AUC by only 1% or 0.5%, respectively. Group-weighted feature selection down to 15, 10, and 5 markers yielded largely different marker panels for both types of co-data used (4 overlapping markers; Table 3) that still performed well in the classification (Figure 4B and Figure 4C). However, the obtained probabilities for CMS3 increased in the true CMS2 samples whereas they decreased in the true CMS3 samples when the number of markers was reduced (Figure 4D). The methylation levels of all selected probes for classification between CMS2 and 3 are depicted in supplementary figure 3A (MATCH-cohort) and supplementary figure 3B (TCGA-cohort).

Table 3. Selected probes

					CpG codata			CMSori codata		
probe_ID	**gene**	**chr**	**position (bp)**	**gene-CpG**	**15m**	**10m**	**5m**	**15m**	**10m**	**5m**
cg19335412	ACTA2	10	90694875	3'UTR-opensea				+		
cg23928468	SLC5A6	2	27433191	5'UTR-shore				+		
cg05951860	CTTNBP2	7	117513101	Body-island				+		
cg20698769	CTTNBP2	7	117513002	Body-island				+	+	+
cg17477990	PDE4DIP	1	144937317	Body-opensea	+	+	+			
cg11125249	GYG1	3	148737622	Body-opensea	+					
cg02827572	C6orf106	6	34566245	Body-opensea	+					
cg04739880	ANKS1A	6	35017865	Body-opensea	+	+				
cg00512872	CYTH3	7	6268584	Body-opensea	+	+	+			
cg14754494	DDC	7	50560743	Body-opensea	+	+		+	+	+
cg19107055	DDC	7	50560686	Body-opensea	+	+	+	+	+	+
cg05357660	PREP	6	105750581	Body-opensea				+	+	
cg23045908	PDE4B	1	66799419	Body-opensea				+		
cg16708174	RARRES1	3	158430962	Body-opensea				+	+	
cg00901138	CHN2	7	29329370	Body-opensea				+	+	
cg00901574	POFUT1	20	30804997	Body-opensea				+		
cg16477879	ASB1	2	239348171	Body-shelf	+	+	+			
cg23219253	ASAP2	2	9518751	Body-shelf	+	+	+			
cg05211192	MAD1L1	7	2119076	Body-shelf	+					
cg12492273	MAD1L1	7	2119499	Body-shelf	+					
cg16772998	MAD1L1	7	2119116	Body-shelf	+	+				
cg00145955	QPRT	16	29703480	Body-shelf	+	+		+	+	+
cg00097384	QPRT	16	29703459	Body-shelf				+	+	
cg27603796	CTTNBP2	7	117512803	Body-shore				+	+	
cg23418465		3	126239121	IGR-shelf	+					
cg17842966	FCGBP	19	40441469	TSS1500-opensea	+	+		+	+	+

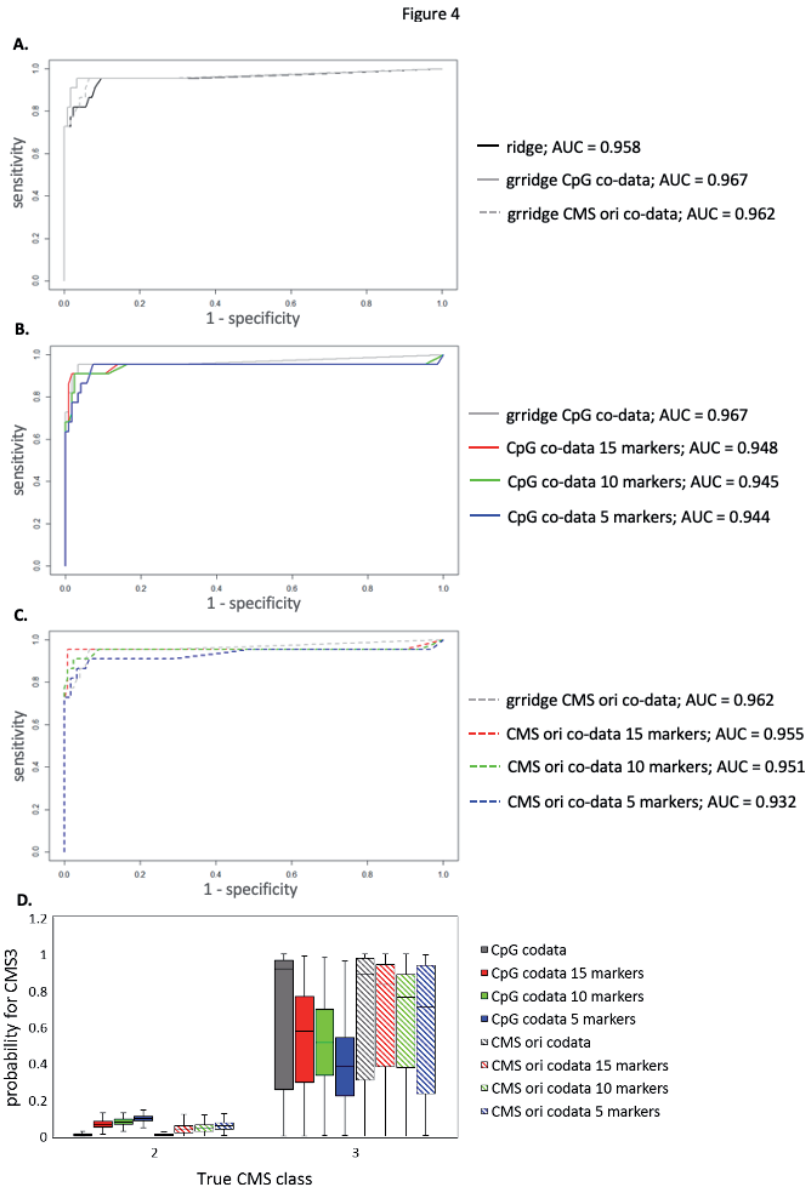


Figure 4. Evaluation of the (gr)ridge prediction models in the training dataset (MATCH)
Receiver-operator curves (ROC) are shown for A. ordinary ridge (black) and group-regularized ridge (grridge) models with CpG co-data (grey) and CMS ori co-data (grey dashed line), B. grridge models based on CpG co-data with post-hoc group-weighted elastic net feature selection of 15 (red), 10 (green), and 5 (blue) markers, and C. grridge models based on CMS ori co-data with post-hoc group-weighted elastic net feature selection of 15 (red), 10 (green), and 5 (blue) markers. In D. the obtained probabilities for CMS3 are plotted for CpG co-data (solid fill) and CMS ori co-data (striped fill) models with all features (grey), 15 markers (red), 10 markers (green), and 5 markers (blue).

Table 4. Performance of both marker panels in MATCH cohort en TCGA cohort

	Model used	CpG		CMSori	
No of probes	Dataset	MATCH	TCGA	MATCH	TCGA
15	Cut off (Youden's index in MATCH data)	0.24/0.25		0.16	
	TNR (spec)	0,98	0,99	0,99	0,98
	TPR (sens)	0,91	0,82	0,95	0,95
	Accuracy	0,97	0,96	0,99	0,98
10	Cut off (Youden's index in MATCH data)	0,21/0,22		0,13	
	TNR (spec)	0,98	0,98	0,98	0,94
	TPR (sens)	0,91	0,82	0,91	0,95
	Accuracy	0,97	0,96	0,97	0,94
5	Cut off (Youden's index in MATCH data)	0,14		0,11	
	TNR (spec)	0,93	0,92	0,94	0,92
	TPR (sens)	0,95	0,91	0,91	0,91
	Accuracy	0,93	0,91	0,93	0,92

The Youden's index was calculated to determine the optimal cut off for the marker panels based on either the CpG co-data and or the CMSori co-data separately. Even when only 5 markers are used, sensitivities, specificities and accuracies >90% are observed for both co-data marker panels in the MATCH dataset using the Youden's index as a cut-off (Table 4).

The obtained models were subsequently fixed and applied to the CMS2 and CMS3 samples from the TCGA dataset, to verify the models' efficacy to predict CMS3 status in independent samples. Using the optimal cut offs selected in the MATCH dataset, the highest performance was established with the 15 marker panels. Again, even the 5 marker panels yielded sensitivities, specificities and accuracies >90% in the TCGA dataset as well (Table 4).

Correlation between DNA methylation levels and RNA expression

To determine the potential impact of the observed methylation patterns on gene expression, we calculated the Spearman correlation between DNA methylation levels and RNA expression for all gene-associated methylation probes in the MATCH cohort. In total, Spearman correlations were determined for 24904 probes. Of these probes, 10.9% and 25.6% were significantly positively and negatively correlated with the expression of their associated gene, respectively. Together, our marker panels included 26 probes of which 25 were associated with a gene. We observed that 28% and 36% were significantly positively and negatively correlated to the expression of their associated gene, respectively (Table 5). For the CMSori co-data marker panel, selection of gene-associated probes was favored by the co-data itself (probes associated with genes included in the CMS SSP classifier), which resulted in 26.7% of the markers showing

Table 5. Correlation between methylation levels and expression levels

probe	gene-CpG	ENSG	gene symbol	CpG-codata	CMSori-codata	Spearman's Rho	FDR
cg23928468	5'UTR-shore	ENSG00000138074	SLC5A6	no	yes	-0.56	2.83E-12
cg00901574	Body-opensea	ENSG00000101346	POFUT1	no	yes	-0.55	1.34E-11
cg00512872	Body-opensea	ENSG00000008256	CYTH3	yes	no	0.47	3.64E-08
cg17842966	TSS1500-opensea	ENSG00000275395	FCGBP	yes	yes	-0.44	2.16E-07
cg00097384	Body-shelf	ENSG00000103485	QPRT	no	yes	0.42	1.67E-06
cg00145955	Body-shelf	ENSG00000103485	QPRT	yes	yes	0.40	4.44E-06
cg27603796	Body-shore	ENSG00000077063	CTTNBP2	no	yes	-0.34	1.23E-04
cg16708174	Body-opensea	ENSG00000118849	RARRES1	no	yes	-0.34	1.43E-04
cg20698769	Body-island	ENSG00000077063	CTTNBP2	no	yes	-0.34	1.91E-04
cg23045908	Body-opensea	ENSG00000184588	PDE4B	no	yes	-0.30	1.15E-03
cg04739880	Body-opensea	ENSG00000064999	ANKS1A	yes	no	0.27	3.76E-03
cg19107055	Body-opensea	ENSG00000132437	DDC	yes	yes	0.25	9.67E-03
cg17477990	Body-opensea	ENSG00000178104	PDE4DIP	yes	no	-0.24	1.42E-02
cg00901138	Body-opensea	ENSG00000106069	CHN2	no	yes	0.24	1.46E-02
cg05951860	Body-island	ENSG00000077063	CTTNBP2	no	yes	-0.21	3.06E-02
cg16477879	Body-shelf	ENSG00000065802	ASB1	yes	no	0.21	3.42E-02
cg19335412	3'UTR-opensea	ENSG00000107796	ACTA2	no	yes	0.14	1.70E-01
cg12492273	Body-shelf	ENSG00000002822	MAD1L1	yes	no	0.14	1.96E-01
cg23219253	Body-shelf	ENSG00000151693	ASAP2	yes	no	-0.13	2.30E-01
cg14754494	Body-opensea	ENSG00000132437	DDC	yes	yes	0.11	3.10E-01
cg11125249	Body-opensea	ENSG00000163754	GYG1	yes	no	-0.05	7.09E-01
cg16772998	Body-shelf	ENSG00000002822	MAD1L1	yes	no	-0.05	7.12E-01
cg02827572	Body-opensea	ENSG00000196821	C6orf106	yes	no	-0.04	7.40E-01
cg05211192	Body-shelf	ENSG00000002822	MAD1L1	yes	no	0.04	7.41E-01
cg05357660	Body-opensea	ENSG00000085377	PREP	no	yes	-0.01	9.32E-01

positive correlation and 53.3% showing negative correlation. In contrast, for the CpG- co-data marker panel we observed that 35.7% of gene-associated markers were positively correlated with expression, whereas only 14.3% were negatively correlated. Compared to all probes (n = 24879) not included in our marker panels, we found that the CpG- co-data marker panel was significantly enriched for positively correlated probes (chi-square test; p = 0.003), whereas the CMSori co-data marker panel was significantly enriched for positively and negatively correlated probes (chi-square test; p = 0.049 and p = 0.014, respectively).

A DNA methylation-based multiclass CMS prediction model

Although dedicated assays are already available for CMS1 and CMS4, ideally one would prefer to have one affordable and practical CMS classification assay applicable to FFPE. Therefore, we also evaluated the potential of DNA methylation for multiclass prediction of CMS1-4. For this purpose the MATCH and TCGA datasets were combined and split into a training (n=283) and test set (n=141) with balanced CMS class distributions and equal contributions from both cohorts. Results obtained applying the model from the training set to the test set indicate that CMS1, CMS2, and CMS3 can be reliably distinguished based on their DNA methylation profiles (Table 6). CMS4, however, is frequently misclassified as CMS2. Using TCGAbiolinks [25] we found that in the TCGA dataset the estimated tumor purity was significantly lower in CMS4 cases, suggesting a larger stromal contribution in these samples (Kruskal-Wallis test; p = 1.30E-31) [26], which may partly explain the classification difficulties. Together these results indicate that DNA methylation markers are not able to reliably classify colon cancers as CMS4 and that the already described dedicated IHC and qRT-PCR assays appear better suited for this purpose [7, 8].

Table 6. Classification of CMS 1-4 based on DNA methylation profiles

		true CMS class				
		CMS 1	**CMS2**	**CMS3**	**CMS4**	**total**
Predicted	CMS1	29	0	2	1	32
	CMS2	1	78	2	7	88
	CMS3	1	0	10	0	11
	CMS4	1	2	0	7	10
	total	32	80	14	15	141
	Correct (%)	90.63	97.50	71.43	46.67	
	False (%)	9.38	2.50	28.57	53.33	

DISCUSSION

In this study, we aimed to identify DNA methylation markers to distinguish between CMS2 and CMS3 in patients with primary CRC based on a genome-wide analysis of DNA methylation in fresh frozen tumor tissues. We showed that CMS2 and CMS3 samples can be distinguished based on overall methylation profiles using subsequent principal component analysis of two independent datasets, and these datasets combined. Group-regularisation of the methylation probes was done based on their location either relative to a CpG island or relative to a gene present in the CMS classifier. This resulted in two different prediction models and subsequently different marker panels. For both panels, even when using only 5 markers, the sensitivity, specificity, and accuracy were >90%. Independent validation of the fixed models in TCGA data showed equal performances. Exploratory multiclass prediction analyses indicate that CMS4 cases are often misclassified as CMS2 based on their DNA methylation profiles.

Thus far, almost all CRC subtyping studies were based on fresh tissue samples, and it remains questionable whether this classification is readily applicable to other types of specimens that are available in the clinic. FFPE-derived RNA is highly degraded and chemically modified, which can impact its utility as a faithful source for classification. Also for the CMS, previous studies have shown that the CMS classifier developed by Guinney et al had a poor performance in FFPE and on biopsy specimen, especially for CMS3 with a specificity of 0.70 [1, 27]. This high type II error rate in CMS3 suggests either biological and/or technical differences between FFPE and fresh-frozen samples and emphasizes the importance of using FFPE samples for training a classifier in this context. Other previous studies have performed DNA methylation analysis of FFPE tissues and provided promising results for the use of FFPE material for DNA methylation profiling [28-31]. Therefore, in contrast with an RNA-based classifier, the methylation panel created in this study is likely to work well on FFPE and may thus provide a promising alternative for use in daily clinical practice.

Correlation analysis has been widely used to examine the relationship between methylation and gene expression. Several studies have elucidated hypermethylation of CpG islands at promoter regions which represses transcription of tumor suppressor genes [32]. However, only one of the probes we identified in both panels was located in the promotor region (within 1500 bp upstream of TSS) of the nearby gene, whereas, except for one intergenic probe, all other probes were located in gene bodies. This is in line with previous research which showed the impact of DNA methylation at intergenic regions and gene bodies on gene expression [33, 34]. DNA methylation in gene body CpG-islands shows an apparent intriguing positive correlation between methylation and gene expression [35, 36]. Yang et al found that from the large amount of methylated probes found in gene body regions, about 20% exhibit a positive correlation between DNA methylation and gene expression. A large proportion of these positively cor-

related genes were overexpressed in primary colon cancer samples compared to normal colon tissues. Our study shows similar results with 28% of the probes from both marker panels being significantly positively correlated to expression of their associated genes. These findings combined, highlight the importance of methylation in gene bodies and warrant further research. Furthermore, our results show that a difference exists between levels of methylation in CMS2 and CMS3 regarding the position of probes relative to CpG-islands. This difference was found for methylation of probes within CpG-island and shores, but not for probes located in shelves or open sea. This is in line with previous research which shows that most tissue-specific DNA methylation and cancer-specific DNA methylation occurs at CpG island shores, especially for colon cancer [37].

Despite the observation that methylation levels in CMS3 were higher in CpG islands and shores compared to CMS2, probes selected by the grridge algorithm as discriminatory panel between CMS2 and CMS3 were actually located in CpG island shelves and in the open sea and mostly showed lower methylation levels in CMS3.

Interestingly, the CMSori- co-data marker panel was enriched for both positively and negatively correlated probes compared to all probes not selected in the panel. This suggests that DNA methylation is at least partly underlying the expression patterns used for the original CMS2 and CMS3 classification. From the selected probes for which methylation and expression were significantly correlated *DDC* expression levels were previously described to vary among colorectal cancer tissues and were associated with disease-free and overall survival [38]. Downregulation of *FCGBP* has been described as a potential target for identification of CRC and lower expression levels also associated with poorer survival within CRC patients [39]. *POFUT1* expression was associated with Notch signalling and decreased goblet cell differentiation and was identified as a potential driver of tumor progression in colorectal adenomas [40]. *PDE4B*, which regulates cellular cAMP concentrations, plays a significant role in regulating the malignant phenotype of CRC cells [41]. *RARRES1* is among the most commonly methylated genes and is silenced in multiple cancers. Interestingly, it is also differentially expressed in metabolism associated diseases [42], supporting a potential role in CMS3 which is featured as the metabolic subtype.

The CMS classification revealed a relatively large number of CMS2 cases and low number of CMS3 cases in the present series. Taking into account the different sample sizes of this study and the original CMS publication, and given the variation in distributions of CMS classes among the six datasets from which the CMS classification originated [1, 7, 43-49] it may be that the CMS class distribution varies per dataset. We chose to use the SSP method for classification because it is not sensitive to the composition of the dataset to which it is applied, so the context of a large series of CRCs or batch effect removal is not required.

Previous literature already provides support for the predictive value of CMS [2]. In addition, new prospective clinical studies are being performed to investigate whether CMS classification can indeed be of added value in clinical decision making by analyzing its predictive value for chemotherapy response [50, 51]. In the future, treatments for colon cancer patients will likely be subtype specific by targeting characteristically overexpressed molecular targets per consensus subgroup [52]. Therefore, a practical, minimally-invasive test to distinguish between the subtypes is needed. Our results show that DNA methylation profiles can be used to discriminate between CMS1, CMS2 and CMS3 cases but does not allow for reliable classification of CMS4. This may due to the relatively large stromal contribution to the CMS4 signature, which is not captured very well in the DNA methylation profile due to the low cell density of stroma. In addition, even though DNA methylation can be used to classify CMS1, we feel that MSI testing, already implemented in routine diagnostics, is more relevant and will capture the vast majority of CMS1 cases [1, 4-6].

For future studies as well as retrospective analyses of archival cohorts, our methylation marker panel should enable the development of a qPCR DNA methylation assay for distinguishing CMS2 from CMS3 in patients with CRC. Such an assay can provide a specific, convenient, and easily implementable tool for use in routine diagnostics. Combined with the already developed assays for CMS1 and CMS4, this assay may accelerate the evaluation of the clinical value of CMS classification and will ultimately help physicians in selecting patients for adjuvant treatment based on their CMS classification.

REFERENCES

1. Guinney J, Dienstmann R, Wang X, et al. The consensus molecular subtypes of colorectal cancer. *Nat Med* 2015; 21(11):1350-6.
2. Song N, Pogue-Geile KL, Gavin PG, et al. Clinical Outcome From Oxaliplatin Treatment in Stage II/III Colon Cancer According to Intrinsic Subtypes: Secondary Analysis of NSABP C-07/NRG Oncology Randomized Clinical Trial. *JAMA Oncol* 2016; 2(9):1162-9.
3. Lindor NM, Burgart LJ, Leontovich O, et al. Immunohistochemistry versus microsatellite instability testing in phenotyping colorectal tumors. *J Clin Oncol* 2002; 20(4):1043-8.
4. Sargent DJ, Marsoni S, Monges G, et al. Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. *J Clin Oncol* 2010; 28(20):3219-26.
5. Des Guetz G, Schischmanoff O, Nicolas P, et al. Does microsatellite instability predict the efficacy of adjuvant chemotherapy in colorectal cancer? A systematic review with meta-analysis. *Eur J Cancer* 2009; 45(10):1890-6.
6. Committee EG. eUpdate - Early Colon Cancer Treatment Recommendations. 2019.
7. Trinh A, Trumpi K, De Sousa EMF, et al. Practical and Robust Identification of Molecular Subtypes in Colorectal Cancer by Immunohistochemistry. *Clin Cancer Res* 2017; 23(2):387-398.
8. Ubink I, Elias SG, Moelans CB, et al. A Novel Diagnostic Tool for Selecting Patients With Mesenchymal-Type Colon Cancer Reveals Intratumor Subtype Heterogeneity. *J Natl Cancer Inst* 2017; 109(8).
9. Feinberg AP, Tycko B. The history of cancer epigenetics. *Nat Rev Cancer* 2004; 4(2):143-53.
10. Esteller M. Epigenetic changes in cancer. *F1000 Biol Rep* 2011; 3:9.
11. Puccini A, Berger MD, Naseem M, et al. Colorectal cancer: epigenetic alterations and their clinical implications. *Biochim Biophys Acta Rev Cancer* 2017; 1868(2):439-448.
12. Van Rijnsoever M, Elsaleh H, Joseph D, et al. CpG island methylator phenotype is an independent predictor of survival benefit from 5-fluorouracil in stage III colorectal cancer. *Clin Cancer Res* 2003; 9(8):2898-903.
13. Shen L, Catalano PJ, Benson AB, 3rd, et al. Association between DNA methylation and shortened survival in patients with advanced colorectal cancer treated with 5-fluorouracil based chemotherapy. *Clin Cancer Res* 2007; 13(20):6093-8.
14. Glockner SC, Dhir M, Yi JM, et al. Methylation of TFPI2 in stool DNA: a potential novel biomarker for the detection of colorectal cancer. *Cancer Res* 2009; 69(11):4691-9.
15. Yang Q, Huang T, Ye G, et al. Methylation of SFRP2 gene as a promising noninvasive biomarker using feces in colorectal cancer diagnosis: a systematic meta-analysis. *Sci Rep* 2016; 6:33339.
16. Chen WD, Han ZJ, Skoletsky J, et al. Detection in fecal DNA of colon cancer-specific methylation of the nonexpressed vimentin gene. *J Natl Cancer Inst* 2005; 97(15):1124-32.
17. Chen JJ, Wang AQ, Chen QQ. DNA methylation assay for colorectal carcinoma. *Cancer Biol Med* 2017; 14(1):42-49.
18. Kloosterman WP, Coebergh van den Braak RRJ, Pieterse M, et al. A Systematic Analysis of Oncogenic Gene Fusions in Primary Colon Cancer. *Cancer Res* 2017; 77(14):3814-3822.
19. Smid M, Coebergh van den Braak RRJ, van de Werken HJG, et al. Gene length corrected trimmed mean of M-values (GeTMM) processing of RNA-seq data performs similarly in

intersample analyses while improving intrasample comparisons. *BMC Bioinformatics* 2018; 19(1):236.
20. Morris TJ, Butcher LM, Feber A, et al. ChAMP: 450k Chip Analysis Methylation Pipeline. *Bioinformatics* 2014; 30(3):428-30.
21. Tian Y, Morris TJ, Webster AP, et al. ChAMP: updated methylation analysis pipeline for Illumina BeadChips. *Bioinformatics* 2017; 33(24):3982-3984.
22. van de Wiel MA, Lien TG, Verlaat W, et al. Better prediction by use of co-data: adaptive group-regularized ridge regression. *Stat Med* 2016; 35(3):368-81.
23. Novianti PW, Snoek BC, Wilting SM, et al. Better diagnostic signatures from RNAseq data through use of auxiliary co-data. *Bioinformatics* 2017; 33(10):1572-1574.
24. Vincent M, Hansen NR. Sparse group lasso and high dimensional multinomial classification. *Computational Statistics & Data Analysis* 2014; 71:771-786.
25. Aran D, Sirota M, Butte AJ. Systematic pan-cancer analysis of tumour purity. *Nature Communications* 2015; 6(1):8971.
26. Colaprico A, Silva TC, Olsen C, et al. TCGAbiolinks: an R/Bioconductor package for integrative analysis of TCGA data. *Nucleic Acids Research* 2015; 44(8):e71-e71.
27. Alderdice M, Richman SD, Gollins S, et al. Prospective patient stratification into robust cancer-cell intrinsic subtypes from colorectal cancer biopsies. *J Pathol* 2018; 245(1):19-28.
28. de Ruijter TC, de Hoon JP, Slaats J, et al. Formalin-fixed, paraffin-embedded (FFPE) tissue epigenomics using Infinium HumanMethylation450 BeadChip assays. *Lab Invest* 2015; 95(7):833-42.
29. Gu H, Bock C, Mikkelsen TS, et al. Genome-scale DNA methylation mapping of clinical samples at single-nucleotide resolution. *Nat Methods* 2010; 7(2):133-6.
30. Li Q, Li M, Ma L, et al. A method to evaluate genome-wide methylation in archival formalin-fixed, paraffin-embedded ovarian epithelial cells. *PLoS One* 2014; 9(8):e104481.
31. Ludgate JL, Wright J, Stockwell PA, et al. A streamlined method for analysing genome-wide DNA methylation patterns from low amounts of FFPE DNA. *BMC Med Genomics* 2017; 10(1):54.
32. Jones PA, Baylin SB. The epigenomics of cancer. *Cell* 2007; 128(4):683-92.
33. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet* 2012; 13(7):484-92.
34. Murtha M, Esteller M. Extraordinary Cancer Epigenomics: Thinking Outside the Classical Coding and Promoter Box. *Trends Cancer* 2016; 2(10):572-584.
35. Yang X, Han H, De Carvalho DD, et al. Gene body methylation can alter gene expression and is a therapeutic target in cancer. *Cancer Cell* 2014; 26(4):577-90.
36. Jjingo D, Conley AB, Yi SV, et al. On the presence and role of human gene-body DNA methylation. *Oncotarget* 2012; 3(4):462-74.
37. Irizarry RA, Ladd-Acosta C, Wen B, et al. The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. *Nat Genet* 2009; 41(2):178-186.
38. Kontos CK, Papadopoulos IN, Fragoulis EG, et al. Quantitative expression analysis and prognostic significance of L-DOPA decarboxylase in colorectal adenocarcinoma. *Br J Cancer* 2010; 102(9):1384-90.
39. Yang W, Shi J, Zhou Y, et al. Integrating proteomics and transcriptomics for the identification of potential targets in early colorectal cancer. *Int J Oncol* 2019; 55(2):439-450.

40. Komor MA, de Wit M, van den Berg J, et al. Molecular characterization of colorectal adenomas reveals POFUT1 as a candidate driver of tumor progression. *International Journal of Cancer* 2020; 146(7):1979-1992.
41. Kim DU, Kwak B, Kim S-W. Phosphodiesterase 4B is an effective therapeutic target in colorectal cancer. *Biochemical and Biophysical Research Communications* 2019; 508(3):825-831.
42. Maimouni S, Issa N, Cheng S, et al. Tumor suppressor RARRES1- A novel regulator of fatty acid metabolism in epithelial cells. *PLoS One* 2018; 13(12):e0208756.
43. De Sousa EMF, Wang X, Jansen M, et al. Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. *Nat Med* 2013; 19(5):614-8.
44. Budinska E, Popovici V, Tejpar S, et al. Gene expression patterns unveil a new level of molecular heterogeneity in colorectal cancer. *J Pathol* 2013; 231(1):63-76.
45. Marisa L, de Reynies A, Duval A, et al. Gene expression classification of colon cancer into molecular subtypes: characterization, validation, and prognostic value. *PLoS Med* 2013; 10(5):e1001453.
46. Roepman P, Schlicker A, Tabernero J, et al. Colorectal cancer intrinsic subtypes predict chemotherapy benefit, deficient mismatch repair and epithelial-to-mesenchymal transition. *Int J Cancer* 2014; 134(3):552-62.
47. Sadanandam A, Lyssiotis CA, Homicsko K, et al. A colorectal cancer classification system that associates cellular phenotype and responses to therapy. *Nat Med* 2013; 19(5):619-25.
48. Perez-Villamil B, Romera-Lopez A, Hernandez-Prieto S, et al. Colon cancer molecular subtypes identified by expression profiling and associated to stroma, mucinous type and different clinical behavior. *BMC Cancer* 2012; 12:260.
49. Cao Y, Lin M, Bu Y, et al. p53-inducible long non-coding RNA PICART1 mediates cancer cell proliferation and migration. *Int J Oncol* 2017; 50(5):1671-1682.
50. van den Berg I, van de Weerd S, Roodhart JML, et al. Improving clinical management of colon cancer through CONNECTION, a nation-wide colon cancer registry and stratification effort (CONNECTION II trial): rationale and protocol of a single arm intervention study. *BMC Cancer* 2020; 20(1):776.
51. Ubink I, Bloemendal HJ, Elias SG, et al. Imatinib treatment of poor prognosis mesenchymal-type primary colon cancer: a proof-of-concept study in the preoperative window period (ImPACCT). *BMC Cancer* 2017; 17(1):282.
52. Thanki K, Nicholls ME, Gajjar A, et al. Consensus Molecular Subtypes of Colorectal Cancer and their Clinical Implications. *Int Biol Biomed J* 2017; 3(3):105-111.

Supplementary Data

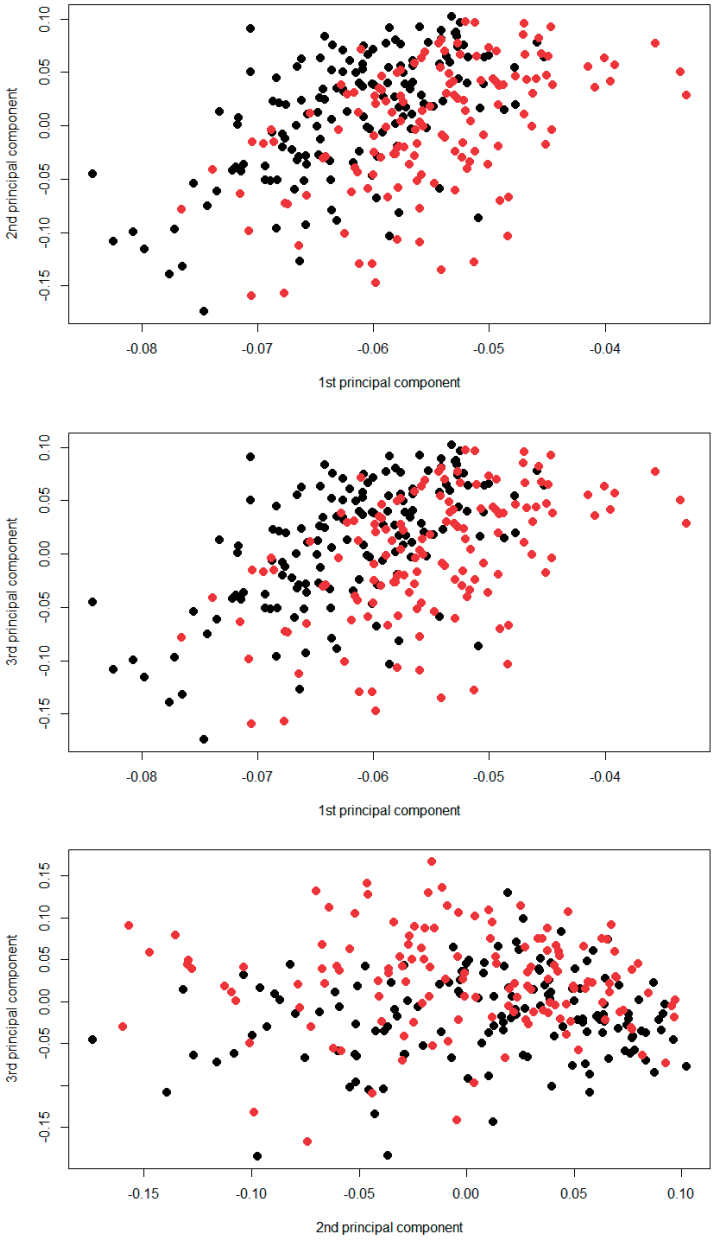
8

Supplementary Table 1. Clinical and histopathological characteristics of CMS2 and CMS3 patients per cohort

	Match							TCGA						
	Total	**(%)**	**CMS 2**	**(%)**	**CMS 3**	**(%)**	**p-value**	**Total**	**(%)**	**CMS 2**	**(%)**	**CMS 3**	**(%)**	**p-value**
	N = 146		**N = 124**	**84.9**	**N = 22**	**15.1**		**N = 140**		**N = 118**	**(84.3)**	**N = 22**	**15.7**	
Gender							0.642							0.810
Male	87	(40.4)	49	(39.5)	10	(45.5)		78	(55.7)	66	(55.9)	10	(45.5)	
Female	59	(59.6)	75	(60.5)	12	(54.5)		60	(42.9)	50	(42.4)	12	(54.5)	
								2	(1.4)	2	(1.7)	0	(0)	
Age (median. IQR)	67 (60-74)		67 (60 – 74)		65.5 (60 – 70)		0.459	66 (55 – 75)		67.5 (56 – 76.8)		60.5 (51.8 – 72.3)		0.139
BMI (median. IQR)	25.8 (23.2 - 28.4)		25.7 (23.4 – 28.4)		25.9 (23.2 – 28.6)		0.808							
Tumor stage							0.503							0.180
I	40	(27.4)	33	(26.6)	7	(31.8)		28	(20)	21	(19.3)	7	(31.8)	
II	63	(43.2)	56	(45.2)	7	(31.8)		48	(34.3)	42	(38.5)	6	(27.3)	
III	43	(29.4)	35	(28.2)	8	(36.4)		35	(25)	27	(24.8)	8	(36.4)	
IV	0	0						20	(14.3)	19	(17.4)	1	(4.5)	
Missing								9	(6.4)					
pT-stage							0.742							0.118
Tis	0	0	0	0	0	(0)		1	(0.7)	0	0	1	(4.5)	
1	0	0	0	0	0	(0)		5	(3.6)	4	(3.4)	1	(4.5)	
2	46	(31.5)	38	(30.6)	8	(36.4)		27	(19.3)	21	(18.1)	6	(27.4)	
3	98	(67.1)	84	(67.7)	14	(63.6)		89	(63.6)	76	(65.6)	13	(59.1)	
4	2	(1.4)	2	(1.6)	0	0		16	(11.4)	15	(12.9)	1	(4.5)	
Missing								2	(1.4)					
pN-stage							0.735							0.363
0	104	(71.2)	90	(72.6)	14	(63.6)		83	(59.3)	70	(60.3)	13	(59.1)	
1	28	(19.2)	22	(17.7)	6	(27.3)		38	(27.2)	30	(25.9)	8	(36.4)	

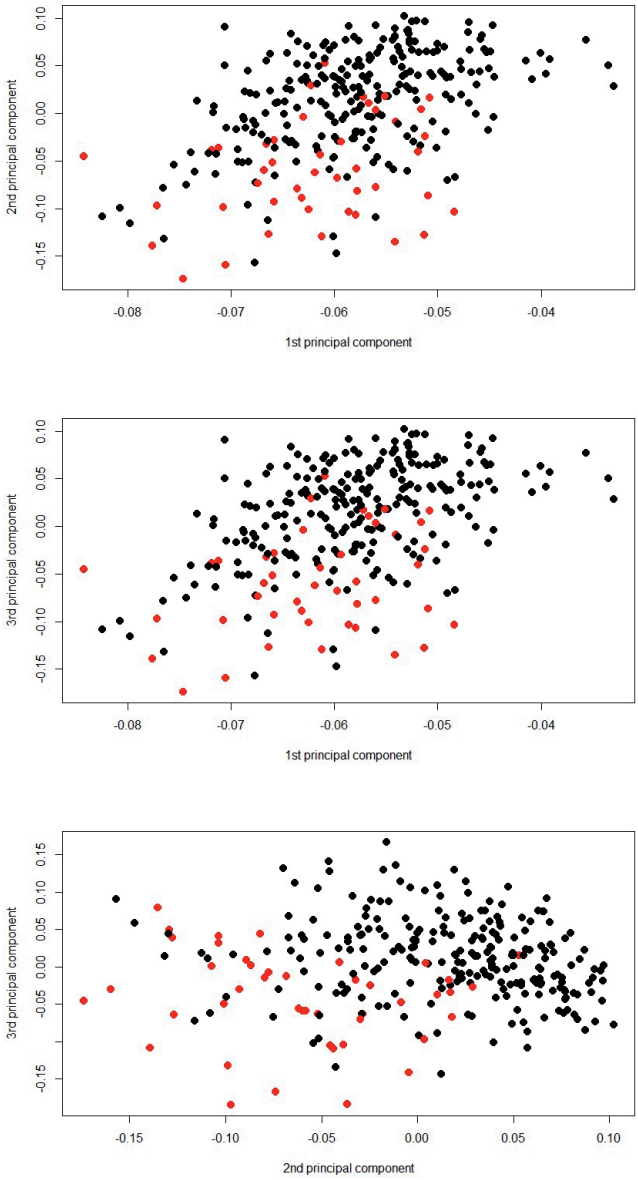
Supplementary Table 1. Clinical and histopathological characteristics of CMS2 and CMS3 patients per cohort (continued)

	Match							TCGA						
	Total	**(%)**	**CMS 2**	**(%)**	**CMS 3**	**(%)**	**p-value**	**Total**	**(%)**	**CMS 2**	**(%)**	**CMS 3**	**(%)**	**p-value**
	N = 146		**N = 124**	**84.9**	**N = 22**	**15.1**		**N = 140**		**N = 118**	**(84.3)**	**N = 22**	**15.7**	
2	14	(9.6)	12	(9.7)	2	(9.1)		17	(12.1)	16	(13.8)	1	(4.5)	
Missing								2	(1.4)					
Tumor differentiation							0.000							
Good	14	(9.6)	14	(11.3)	0	(0.0)								
Moderate	123	(84.2)	107	(86.3)	16	(72.7)								
Poor	8	(5.5)	3	(2.4)	5	(22.8)								
Unknown/Other	1	(0.7)	0	(0)	1	(4.5)								
Tumor location							0.230							0.008
Right	54	(37)	43	(34.7)	11	(50)		71	(50.7)	54	(45.7)	17	(77.3)	
Left	92	(63)	81	(65.3)	11	(50)		61	(43.6)	57	(48.3)	4	(18.2)	
Missing								8	(5.7)	7	(6)	1	(0.05)	
Rectum/Colon														0.751
Colon	146	(100)						137	(97.9)	115	(97.5)	22	(100)	
Rectum	0	0						1	(0.7)	1	(0.8)	0	0	
Missing								2	(1.4)	2	(1.7)	0	0	
Adjuvant therapy							0.446							
No	104	(71.2)	90	(72.6)	14	(63.6)								
Yes	42	(28.8)	34	(27.4)	8	(36.4)								



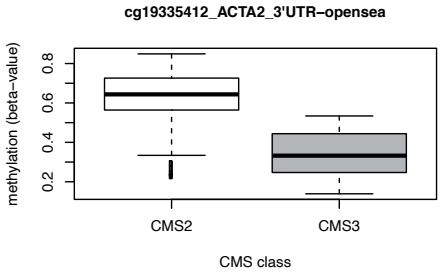
Supplementary Figure 1. Principal Component Analysis (PCA) of DNA methylation profiles from all CMS2 and CMS3 samples present in the MATCH and TCGA cohorts

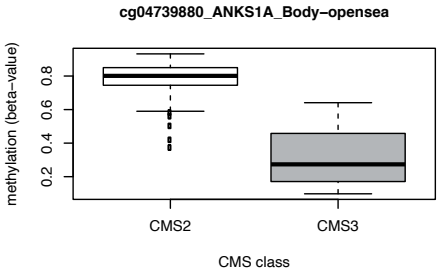
Principal components were calculated for DNA methylation profiles of 286 colorectal cancer tissues (146 from MATCH cohort (black) and 140 from TCGA cohort). In A. PC1 is shown on the X-axis and PC2 on the y-axis. In B. PC1 is shown on the x-axis and PC3 in the y-axis. In C. PC2 is shown on the x-axis and PC3 on the y-axis. Each dot represents 1 sample. Samples are colored based on their cohort of origin (MATCH in black and TCGA in).

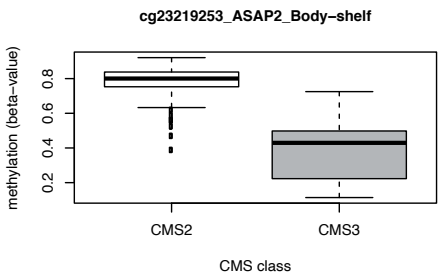


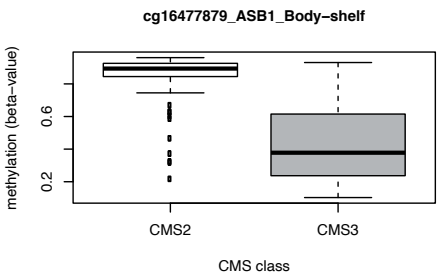
Supplementary Figure 2 – Principal Component Analysis (PCA) of DNA methylation profiles from all CMS2 and CMS3 samples present in the MATCH and TCGA cohorts

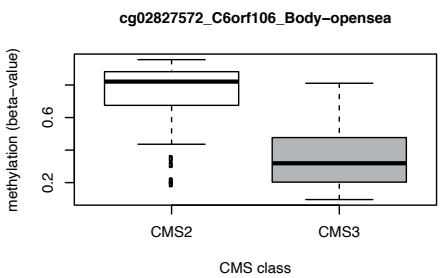
Principal components were calculated for DNA methylation profiles of 286 colorectal cancer tissues (242 CMS2 samples (black) and 44 CMS3 samples). In **A.** PC1 is shown on the X-axis and PC2 on the y-axis. In **B.** PC1 is shown on the x-axis and PC3 in the y-axis. In **C.** PC2 is shown on the x-axis and PC3 on the y-axis. Each dot represents 1 sample. Samples are colored based on CMS classification (CMS2 in black and CMS3).

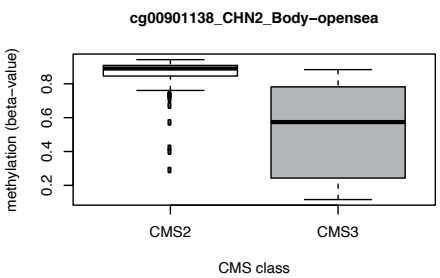

cg19335412_ACTA2_3'UTR–opensea
methylation (beta–value)
0.2
0.4
0.6
0.8
CMS2
CMS3
CMS class


cg04739880_ANKS1A_Body–opensea
methylation (beta–value)
0.2
0.4
0.6
0.8
CMS2
CMS3
CMS class


cg23219253_ASAP2_Body–shelf
methylation (beta–value)
0.2
0.4
0.6
0.8
CMS2
CMS3
CMS class


cg16477879_ASB1_Body–shelf
methylation (beta–value)
0.2
0.6
CMS2
CMS3
CMS class


cg02827572_C6orf106_Body–opensea
methylation (beta–value)
0.2
0.6
CMS2
CMS3
CMS class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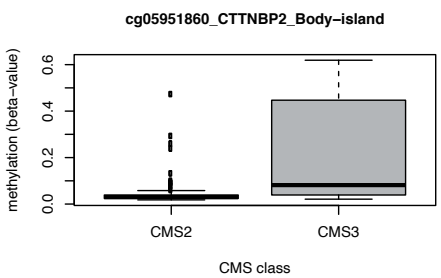
methylation (beta–value)
0.2
0.4
0.6
0.8
CMS2
CMS3
CMS class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methylation (beta–value)
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0.2
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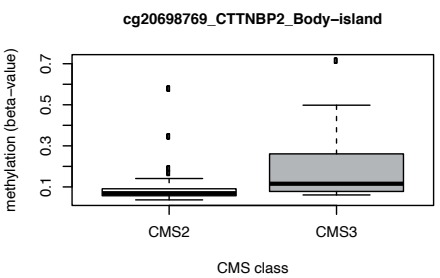
CMS2
CMS3
CMS class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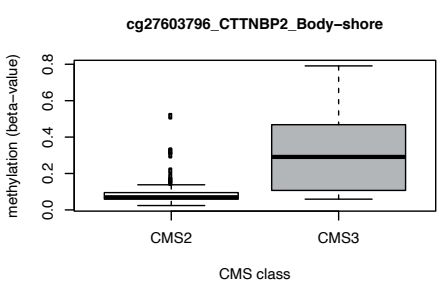
0.5
0.7
CMS2
CMS3
CMS class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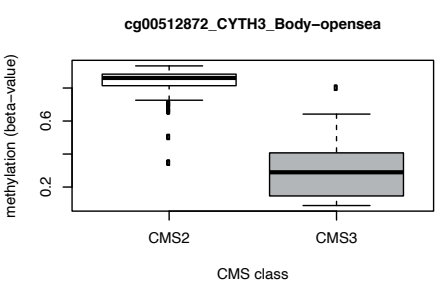
0.2
0.4
0.6
0.8
CMS2
CMS3
CMS class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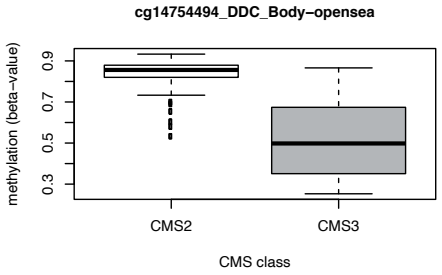
0.6
CMS2
CMS3
CMS class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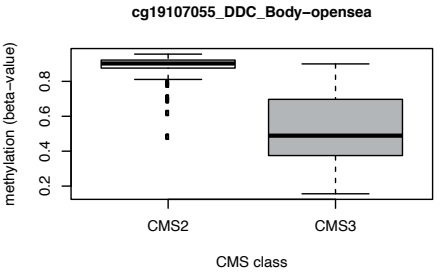
0.5
0.7
0.9
CMS2
CMS3
CMS class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methylation (beta−value)
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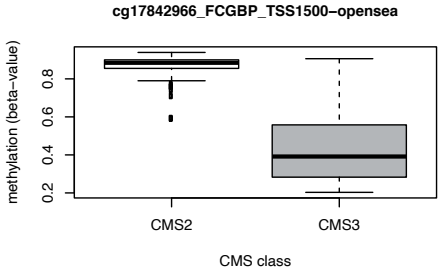
0.4
0.6
0.8
CMS2
CMS3
CMS class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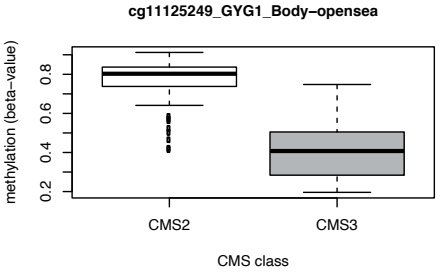
methylation (beta−value)
0.2
0.4
0.6
0.8
CMS2
CMS3
CMS class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methylation (beta−value)
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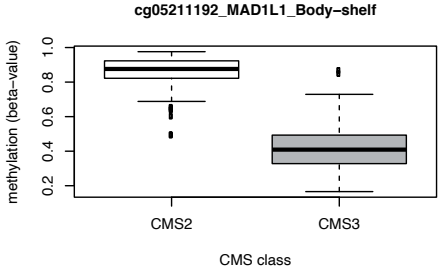
0.6
0.8
CMS2
CMS3
CMS class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0.4
0.6
0.8
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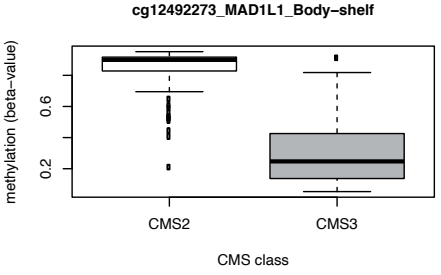
CMS2
CMS3
CMS class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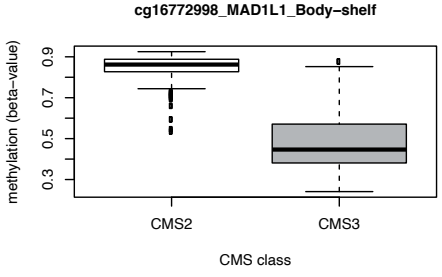
0.6
CMS2
CMS3
CMS class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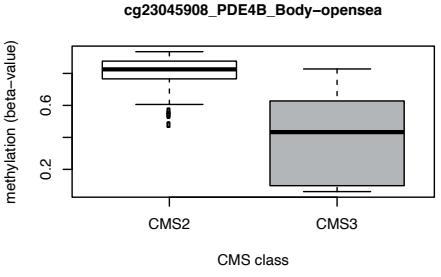
0.5
0.7
0.9
CMS2
CMS3
CMS class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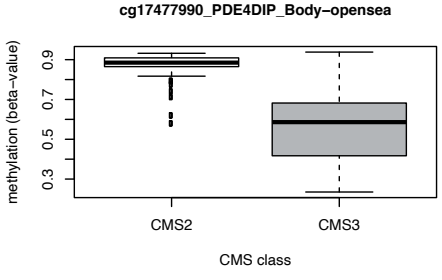
methylation (beta−value)
0.2
0.6
CMS2
CMS3
CMS class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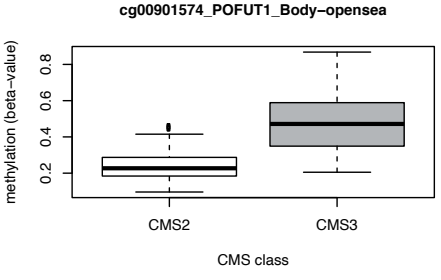
methylation (beta−value)
0.3
0.5
0.7
0.9
CMS2
CMS3
CMS class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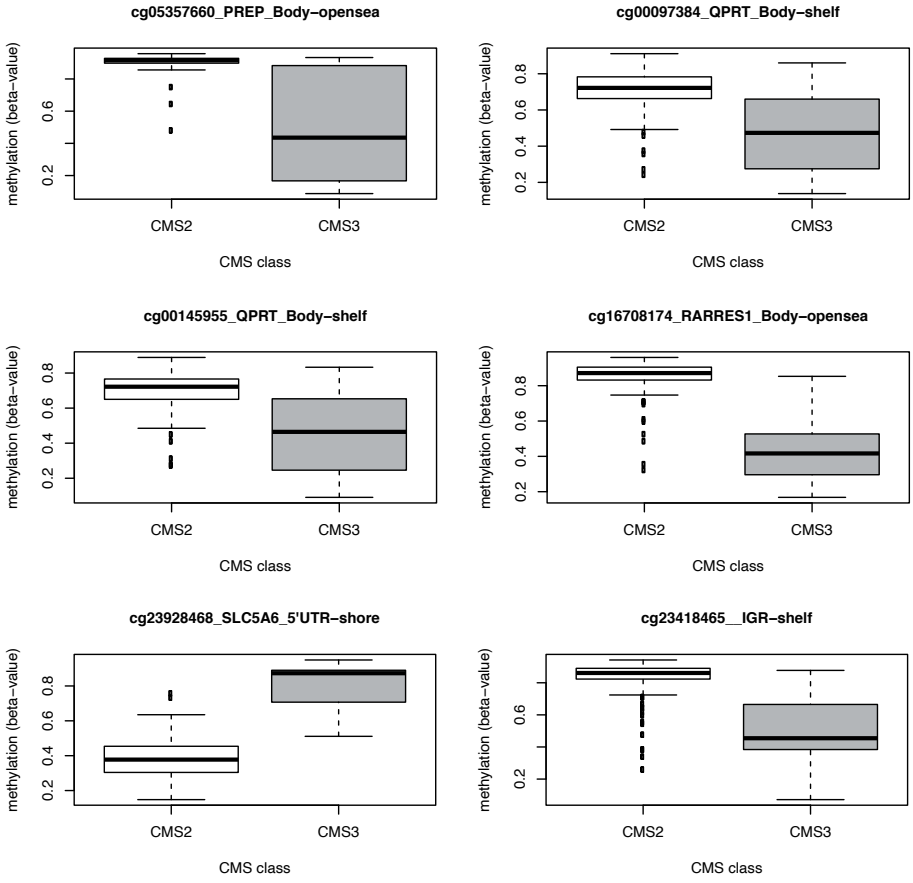
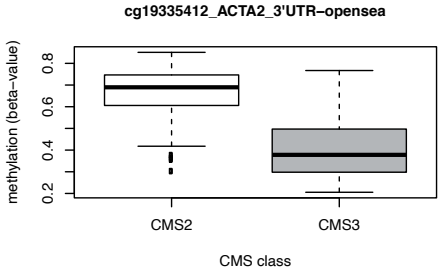
methylation (beta−value)
0.2
0.4
0.6
0.8
CMS2
CMS3
CMS class



Supplementary Figure 3A – The methylation levels of all selected probes for classification between CMS2 and 3 from the MATCH-cohort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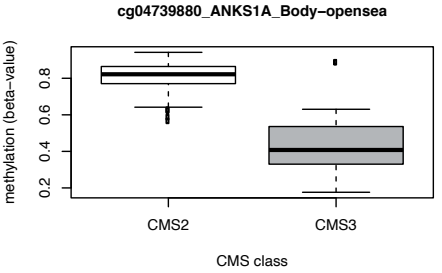
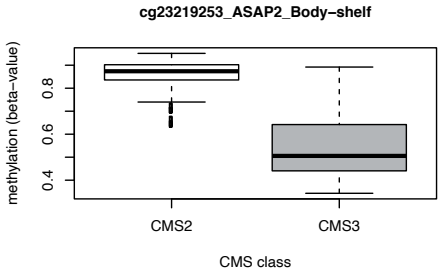
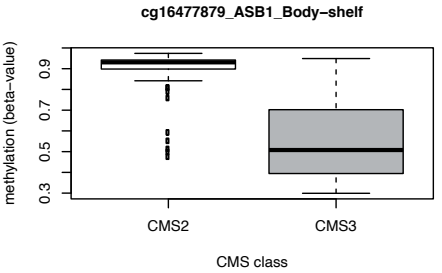
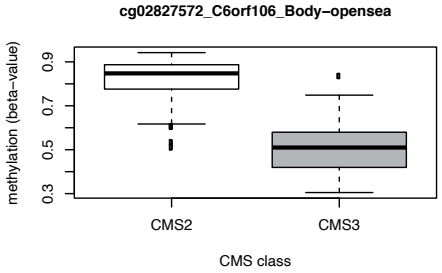
methylation (beta−value)
0.2
0.4
0.6
0.8
CMS2
CMS3
CMS class


cg04739880_ANKS1A_Body−opensea
methylation (beta−value)
0.2
0.4
0.6
0.8
CMS2
CMS3
CMS class


cg23219253_ASAP2_Body−shelf
methylation (beta−value)
0.4
0.6
0.8
CMS2
CMS3
CMS class


cg16477879_ASB1_Body−shelf
methylation (beta−value)
0.3
0.5
0.7
0.9
CMS2
CMS3
CMS class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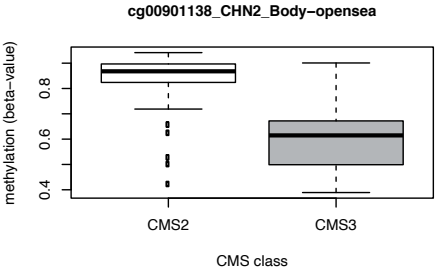
methylation (beta−value)
0.3
0.5
0.7
0.9
CMS2
CMS3
CMS class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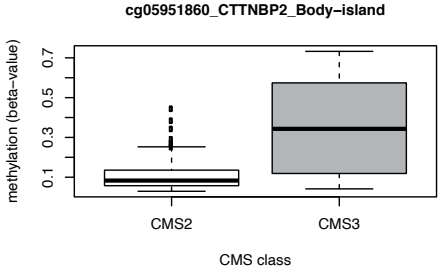
methylation (beta−value)
0.4
0.6
0.8
CMS2
CMS3
CMS class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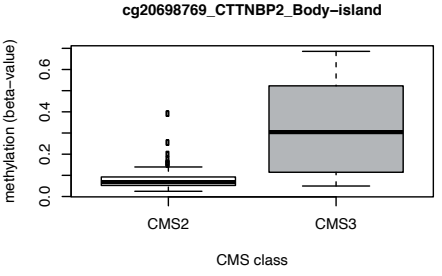
0.3
0.5
0.7
CMS2
CMS3
CMS class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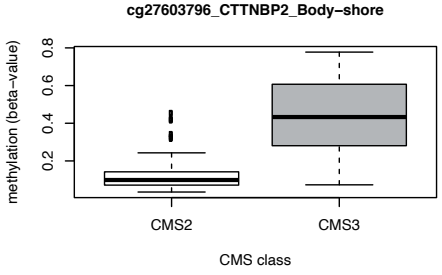
methylation (beta−value)
0.0
0.2
0.4
0.6
CMS2
CMS3
CMS class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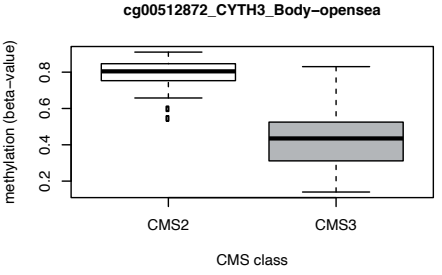
methylation (beta−value)
0.2
0.4
0.6
0.8
CMS2
CMS3
CMS class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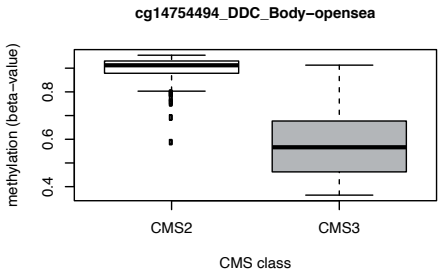
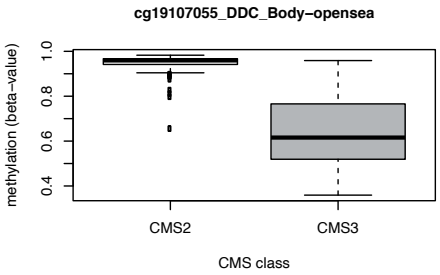
0.6
0.8
CMS2
CMS3
CMS class


cg14754494_DDC_Body−opensea
methylation (beta−value)
0.4
0.6
0.8
CMS2
CMS3
CMS class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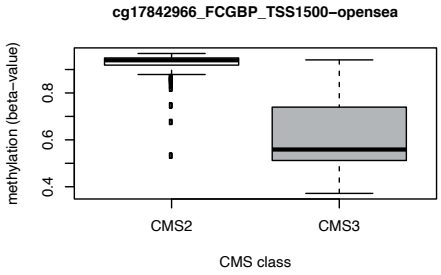
methylation (beta−value)
0.4
0.6
0.8
1.0
CMS2
CMS3
CMS class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methylation (beta−value)
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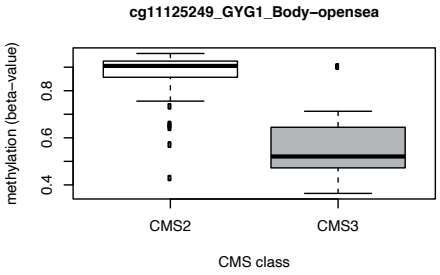
0.6
0.8
CMS2
CMS3
CMS class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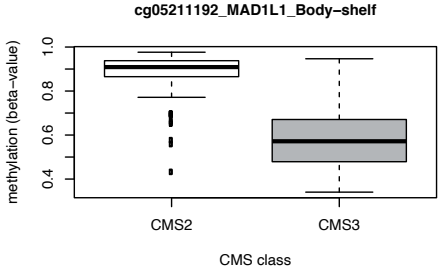
0.6
0.8
CMS2
CMS3
CMS class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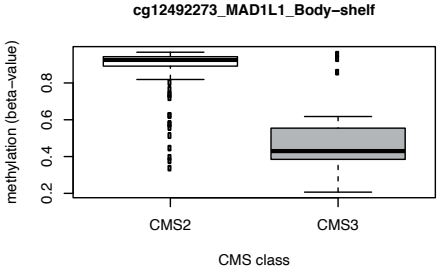
0.6
0.8
1.0
CMS2
CMS3
CMS class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methylation (beta−value)
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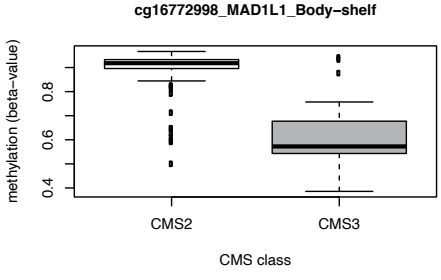
0.4
0.6
0.8
CMS2
CMS3
CMS class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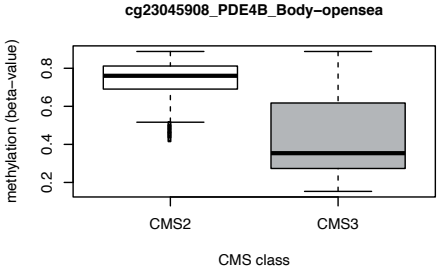
0.6
0.8
CMS2
CMS3
CMS class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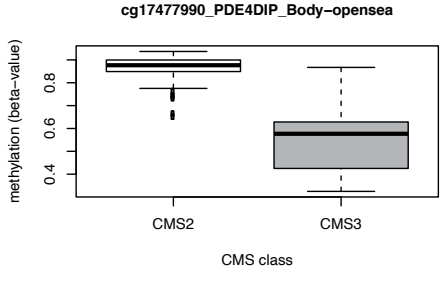
methylation (beta−value)
0.2
0.4
0.6
0.8
CMS2
CMS3
CMS class


cg17477990_PDE4DIP_Body−opensea
methylation (beta−value)
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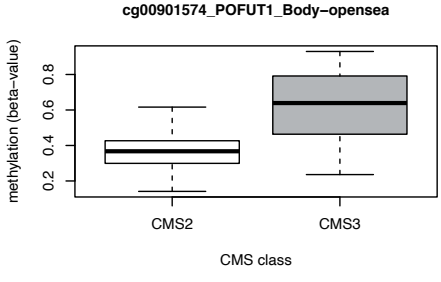
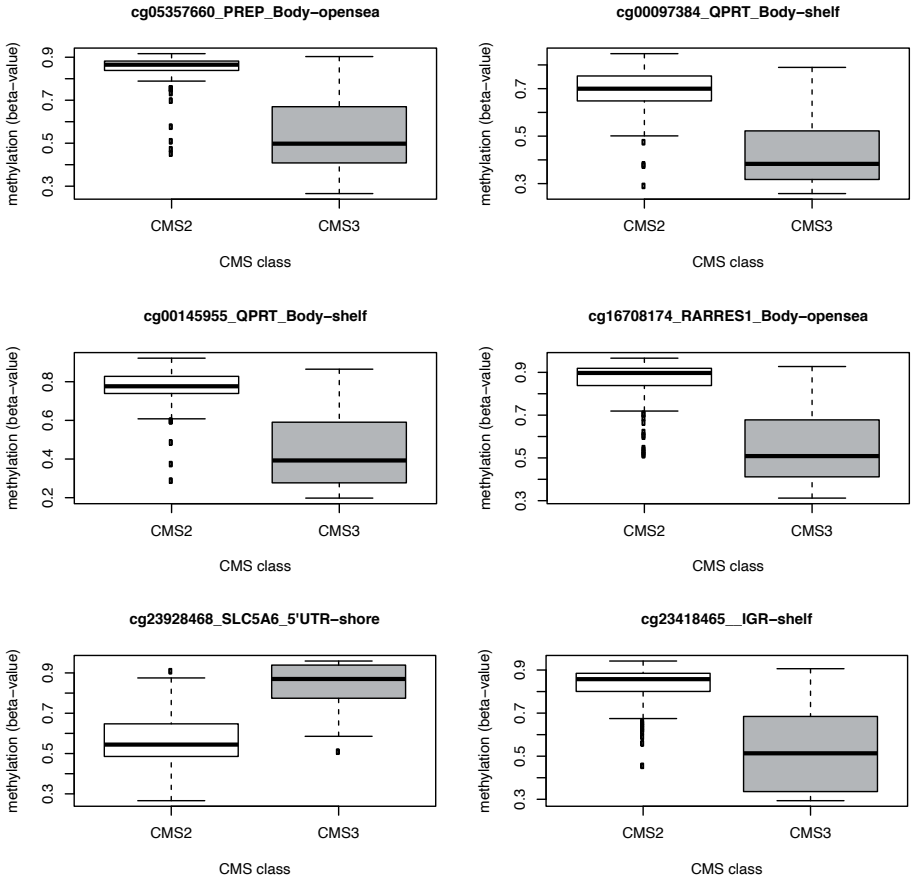
0.6
0.8
CMS2
CMS3
CMS class


cg00901574_POFUT1_Body−opensea
methylation (beta−value)
0.2
0.4
0.6
0.8
CMS2
CMS3
CMS class



Supplementary Figure 3B – The methylation levels of all selected probes for classification between CMS2 and 3 from the TCGA-cohort

Chapter 9

Improving clinical management of colon cancer through CONNECTION, a nation-wide colon cancer registry and stratification effort (CONNECTION II Trial): Rationale and protocol of a single arm intervention study

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ABSTRACT

Background

It is estimated that around 15-30% of patients with early stage colon cancer benefit from adjuvant chemotherapy. We are currently not capable of upfront selection of patients who benefit from chemotherapy, which indicates the need for additional predictive markers for response to chemotherapy.

It has been shown that the consensus molecular subtypes (CMSs), defined by RNA-profiling, have prognostic and/or predictive value. Due to postoperative timing of chemotherapy in current guidelines, tumor response to chemotherapy per CMS is not known, which makes the differentiation between the prognostic and predictive value impossible. Therefore, we propose to assess the tumor response per CMS in the neoadjuvant chemotherapy setting. This will provide us with clear data on the predictive value for chemotherapy response of the CMSs.

Methods

In this prospective, single arm, multicenter intervention study, 262 patients with resectable microsatellite stable cT3-4NxM0 colon cancer will be treated with two courses of neoadjuvant and two courses of adjuvant capecitabine and oxaliplatin. The primary endpoint is the pathological tumor response to neoadjuvant chemotherapy per CMS. Secondary endpoints are radiological tumor response, the prognostic value of these responses for recurrence free survival and overall survival and the differences in CMS classification of the same tumor before and after neoadjuvant chemotherapy. The study is scheduled to be performed in 8-10 Dutch hospitals. The first patient was included in February 2020.

Discussion

Patient selection for adjuvant chemotherapy in early stage colon cancer is far from optimal. The CMS classification is a promising new biomarker, but a solid chemotherapy response assessment per subtype is lacking. In this study we will investigate whether CMS classification can be of added value in clinical decision making by analyzing the predictive value for chemotherapy response. This study can provide the results necessary to proceed to future studies in which (neo)adjuvant chemotherapy may be withhold in patients with a specific CMS subtype, who show no benefit from chemotherapy and for whom possible new treatments can be investigated.

Trial Registration

This study has been registered in the Netherlands Trial Register (NL8177) at 11-26-2019, https://www.trialregister.nl/trial/8177. The study has been approved by the medical ethics committee Utrecht (MEC18/712).

BACKGROUND

Colon cancer is one of the most common types of cancer in the Netherlands with an incidence of around 9.800 patients in 2018 [1]. Approximately 80% of patients present with local disease (stage I-III). Curative surgery followed by adjuvant systemic chemotherapy is standard of care in patients with microsatellite stable (MSS) high-risk stage II and stage III colon cancer. Despite this intensive treatment, 20-30% of the patients develop metastatic disease. These patients do not benefit enough from the current adjuvant systemic therapy. Moreover, it is estimated that 50% will not develop metastases after surgery alone and are therefore over-treated with adjuvant chemotherapy. Identifying the patients at risk of developing metastases, as well as those responding to therapy is a clear unmet need in colon cancer care. The development of new prognostic and predictive markers for chemotherapy response is therefore of utmost importance.

Many efforts have been undertaken to stratify CRC patients into biologically and clinically distinct subtypes. One of these led to the development of the Consensus Molecular Subtypes (CMSs), which is based on RNA expression profiling of tumor tissue and which is currently considered to be the most robust molecular stratification in CRC [2]. CMS1 is characterized by hypermutation, microsatellite instability (MSI) and strong immune infiltration. CMS2, the canonical subtype, has marked WNT and MYC signaling activation. CMS3 is enriched for *KRAS*-mutations and shows evident metabolic deregulation. CMS4, the mesenchymal subtype, is characterized by prominent TGF-β activation, stromal invasion and angiogenesis activation. Subtyping in a large heterogeneous patient cohort (n=2.129) with stage I to IV colorectal cancer showed significant differences in prognosis, with CMS4 as the poor-prognosis subtype, confirming the clinical relevance of the intrinsic processes implicated in each CMS [2].

These results support the idea that the CMSs might have predictive value for response to chemotherapy. Due to the postoperative timing of chemotherapy in current guidelines, the tumor response to chemotherapy is not assessable, which makes a distinction between the prognostic value and predictive value of the subtypes impossible. Only a randomized controlled trial in which patients would be randomized in either surgery plus adjuvant chemotherapy or surgery alone would make this distinction possible. However, this causes ethical dilemmas

because chemotherapy would be withhold in patients who might actually benefit. Yet, a solid response assessment per subtype is necessary for implementation in clinical decision-making. We therefore propose to treat patients with two neoadjuvant and two adjuvant courses of CAPOX and determine the response in tumor resected specimens.

Applying neo-adjuvant chemotherapy may have several advantages: the possibility of response monitoring, early eradication of micrometastases and more complete resections. Neo-adjuvant treatment is already standard of care for different GI malignancies including esophageal, gastric and rectal cancers [3-7]. The FOxTROT Collaborative Group (2012) was the first to set up a neoadjuvant trial in patients with locally advanced resectable colon cancer and concluded that preoperative chemotherapy is feasible with acceptable toxicity and perioperative morbidity [8]. After this pilot study, they conducted a randomized phase 3 trial investigating the effect of neoadjuvant chemotherapy in patients with a cT3-4N0-2M0 colon cancer. Patients were randomized 2:1 between 6 weeks of neoadjuvant combined with 18 weeks of adjuvant FOLFOX/CAPOX or 24 weeks of adjuvant FOLFOX/CAPOX. Neoadjuvant chemotherapy was safe with less major surgical complications, significant down-staging and a reduced risk of incomplete resection. Although the primary endpoint of the study (freedom from recurrent or persistent disease after 2 years) was not met, the risk of a recurrence after 2 years was reduced to 13.6% with peri-operative chemotherapy compared to 17.2% with adjuvant chemotherapy only (HR 0.75 (0.55-1.04), p = 0.08)) [9].

In the proposed study we will investigate the predictive value of the CMS classification on chemotherapy response in a neoadjuvant setting, including pathological response and radiological response and their correlation with RFS and OS. This allows us to determine therapy efficacy in individual patients and per subtype.

Objective

The primary aim of this study is the evaluation of the pathological tumor response to neoadjuvant systemic chemotherapy per CMS in patients with MSS high risk stage II and stage III colon cancer.

METHODS

Study Design

CONNECTION II is a prospective, multicenter interventional cohort study that will be performed as a substudy of the Prospective Dutch ColoRectal Cancer cohort (PLCRC). PLCRC is

a nationwide cohort study of the Dutch Colorectal Cancer Group (DCCG), facilitating scientific research to improve the outcome and quality of life of patients with colorectal cancer [10]. We aim to include patients in 8-10 Dutch hospitals that participate in PLCRC.

In CONNECTION II patients with a MSS cT3-4NxM0 colon tumor will be treated with two courses of neoadjuvant and two courses of adjuvant capecitabine and oxaliplatin (CAPOX) (Figure 1 & Table 1). The CMS classification will be determined on both the pre-treatment biopsies and the resection specimen. At least 4 multi-region biopsies will be taken pre-treatment to ensure a sample with vital tumor and sufficient RNA quality. Tumor response will be assessed on the resection specimen using the tumor response grading (TRG) system as proposed by Dworak et al [11]. Radiological response evaluation will be centrally performed by dedicated radiologists on sequential CT scans made at baseline and after two courses of neoadjuvant chemotherapy but before resection. Pathologists and radiologists will be blinded for the CMS classification.

Optionally, blood samples are taken for circulating tumor DNA (ctDNA) analysis and plasma storage at four time points: at baseline, after neoadjuvant treatment, after surgery and after completion of the adjuvant chemotherapy. Follow-up will be performed until 10 years post-surgery. Data on local recurrences, metastases and survival will be documented.

Study Population

Patients diagnosed with resectable cT3-4NxM0 colon cancer are eligible for the CONNECTION II trial. Baseline CT-scans of all patients will be reviewed by dedicated radiologists in the treating hospitals with special focus on tumor staging. MSI status will be determined on biopsy material to exclude patients with an MSI tumor [14].

Patients are eligible when they meet the following criteria:

- Able and willing to provide written informed consent for the CONNECTION II study
- Informed consent signed for PLCRC components ‘clinical data’ and ‘future studies’
- MSS based on pre-treatment biopsy by immunohistochemistry (IHC)
- Fit to undergo neoadjuvant chemotherapy with capecitabine + oxaliplatin and subsequent surgery judged by the primary treating physician
- Adequate bone marrow, liver and renal function

Patients will be excluded if any of the following criteria are met:

- Any other malignant disease within the preceding 5 years apart from non-melanotic skin cancer, carcinoma in situ and early stage disease with a recurrence risk of less than 5%
- Colonic obstruction that cannot be defunctioned by a stoma
- Pregnant or lactating women

Main Study Parameter/Endpoint

The primary endpoint is the pathological tumor response to neoadjuvant chemotherapy per CMS. The pathological response will be centrally scored on HE-stained slides from the resection specimen using the tumor response grading system according to Dworak [15,16]. Based on results from the FOxTROT study, a good response will be defined as TRG2, TRG3, or TRG4; poor response as TRG1, or TRG0. The CMS classification will be determined on the pre-treatment biopsies and on the resection specimens. RNA will be isolated from FFPE material and analyzed on the nCounter SPRINT profiler, a reliable and robust platform for samples with degraded RNA such as FFPE samples [12-14].

Secondary study parameters/endpoints

- Additional pathological markers to assess the tumor response: the modified Ryan scheme (TRS) [15] and expression of Ki-67 and Caspase-3 and morphological cytostatic-cytotoxic effects on HE-stained tissue slides
- Pathological response per TRG and TRS category separately for the different CMS subtypes
- Radiological tumor response to neoadjuvant chemotherapy
- Recurrence free survival (RFS) at two and three years. RFS is defined as the time elapsed between the diagnosis of the primary tumour and either the date of any recurrence of disease, time of death, or the date of the last follow-up visit at which a patient was considered to have no recurrence.
- Overall survival (OS) at five and ten years
- Therapy-induced CMS differences.
- Prognostic and predictive value of cytotoxic lymphocytes (CytoLym) and cancer-associated fibroblasts (CAF) infiltration scores.
- Diagnostic accuracy of ctDNA measurements for monitoring treatment response to neoadjuvant treatment and detection of residual disease.
- Exploration of proteome profiles for monitoring treatment response to neoadjuvant treatment and detection of residual disease.
- Percentages of pathological complete (R0), pathological microscopic incomplete (R1) and pathologically macroscopic incomplete (R2) resections.
- Surgical complication rate (i.e. wound infections and anastomotic leak)

Statistical Analysis

Primary study endpoint

The primary study endpoint is the pathological tumor response per CMS using the TRG system according to Dworak [11]. Pathologic tumor regression rates with corresponding 95% confidence intervals will be analyses per CMS subgroup using the Wilson Method.

Secondary study endpoints

Categorical data (pathological tumor response according to the Modified Ryan scheme) are compared using Chi-square analysis or Fisher's exact test and are shown as numbers, relative and absolute rates. Continuous data (CytoLym and CAF infiltration scores, radiological tumor response, pathological response by percentage of Ki-67 and Caspase-3 positive neoplastic cells) are compared using non-parametric T-test or Mann-Whitney U test where appropriate and are shown as mean and standard deviation or median and interquartile range (25%-75%). P-values are two-tailed and results <0.05 are considered significant.

The OS at 5 and 10 years and RFS at 2 and 3 years will be calculated and depicted by means of the Kaplan Meier technique and will be compared using the (stratified) logrank test. Hazard ratios and 95% confidence intervals will be calculated with a (stratified) cox-proportional hazard analysis. The RFS will be analyzed per CMS subgroup, per TRG and radiological response. All estimates will be accompanied by 95% confidence intervals.

Sample size calculation

We based our sample size calculation on the desired precision with which we will be able to estimate the pathological response rates to neoadjuvant chemotherapy within each CMS subtype. This precision is quantified by the margin of error (the radius of the 95% confidence interval), which we set at a maximum of 15%. This margin of error is achieved with 35 patients in the least prevalent CMS subgroup, namely CMS3, and an anticipated 11 pathologic responses, yielding a response rate of 31% with a 95%CI of 19-48%. Based on the currently observed ratios of subtypes derived from the large consensus dataset after exclusion of the MSI tumors (which holds CMS1 tumors for most part) we will need a total of 209 MSS patients (CMS2 49%, CMS3 17%, CMS4 35%). With this sample size we anticipate maximum margins of error of 8.9%, 14.7%, and 10.3% for CMS2, CMS3, and CMS4 respectively, and 6.2% overall.

The above depends on the assumption that the response rates will not be higher than ~30% within each CMS subgroup. If response rates will actually be closer to 50%, the maximum margin of error will increase.

The sample size hence indicates that for the analysis, 209 patients will be needed for whom follow-up and subtype is known. We expect a 25% loss in patients due to loss of follow-up, insufficient quality of the biopsy material or failure to faithfully assign patients to a subgroup based on the RNA expression profiles resulting in a total of 262 patients needed to have sufficient data for both the primary and secondary outcomes.

Data collection and data management

Data collection and data management will be performed by the Netherlands Comprehensive Cancer Organization (IKNL). They have broad experience with continuous data collection based on high quality electronic case report forms (eCRFs) which guarantees complete and timely recording, handling and storage of data and documents. All local and central data managers are registered and the electronic database (TRIAS) is ISO certified. Data will be documented in line with 'Good Clinical Practice (GCP)' and Dutch legal requirements. Major violations of the protocol will be recorded.

Monitoring

No data and safety monitoring board (DSMB) will be assigned, since patients are subjected to an intervention with a low postoperative morbidity that is already being performed in routine clinical practice. No interim analyses will be performed.

Auditing

Independent monitoring of the study is performed by a qualified monitor of IKNL. The monitor plan is based on the judgement of the IRB that study participation is of low to moderate risk. Monitoring will be performed by investigating the electronic trial database and performing site visits. Each participating site will be visited at least once, with repeat visits to sites where performance is a concern. The quality assessment will focus on the safety, wellbeing and rights of the patients, the quality of the documented data in the eCRF and their traceability to source documents and the completeness of the regulatory binder. After each monitor visit, the trial monitor reports feedback to the project leader, study coordinator and local investigator.

Adverse events

The treatment with CAPOX in this study is standard of care, therefore AE and SAE are not expected to be different. As both the treatment with CAPOX and the surgery are part of the standard of care, only two specific SAEs are defined which are possibly related to the adjusted study schedule. Information will be collected on patients who prematurely stop chemotherapy

treatment and of patients who are not able to undergo planned surgery due to progressive disease/obstruction.

The following two SAEs will be reported:

- If the surgery has to be postponed for more than 8 weeks after the start of cycle 2 of CAPOX
- If patients can not complete all the neo-adjuvant chemotherapy courses

The study coordinator will report these SAEs to the accredited Institutional Review Board (IRB) that approved the study protocol.

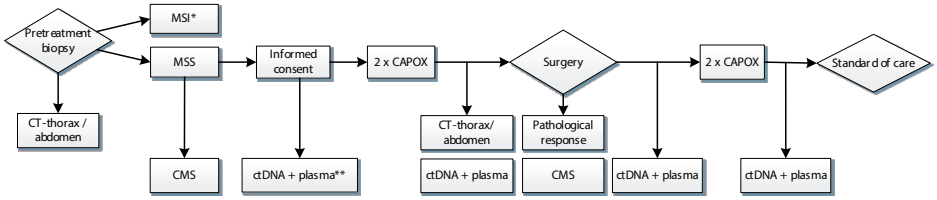

Figure 1. Flow diagram of clinical study. *Patients with an MSI tumor will be excluded from this study. ** At 4 time points blood samples will be collected for ctDNA analyses and future biomarker studies.

DISCUSSION

Colon cancer is one of the most common types of cancer in the Netherlands. The standard of care for patients with MSS high risk stage II and stage III colon cancer currently consists of surgery followed by systemic chemotherapy. Patient selection for adjuvant chemotherapy is still far from optimal. Approximately 50% would never develop metastases after surgery alone and is therefore over-treated with adjuvant chemotherapy. Moreover, 20-30% still develop metastatic disease despite this intensive treatment, leaving merely 15-30% that in fact benefit from adjuvant chemotherapy. This illustrates the evident need for additional predictive markers for chemotherapy benefit.

One potential marker is the CMS classification, which is based on the integration of six different molecular classification systems based on RNA expression profiling. The CMS classification divides CRC patients into four subtypes with distinctive biological features. Guinney et al. showed a clear relapse free survival and overall survival advantage for CMS1-3 compared to CMS4 in a heterogeneous patient cohort with stage I-IV CRC with divergent treatment schemes [2].

Table 1. Study flowchart of clinical study

Study procedures	**Inclusion**	**Neo-adjuvant Chemotherapy**						**Surgery**				**Adjuvant Chemotherapy**						**Follow up**			
week	<0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Check in- and exclusion	x																					
Sign Informed Consent	x																					
Blood withdrawal for ctDNA + plasma	*x* [a]							*x* [b]				*x* [c]									*x* [d]	
CT-scan	x							*x* [f]														
Surgery									x[g]													
CAPOX		*C1D1*			*C2D1*								*C3D1* [e]			*C4D 1*						
Record medical history	x	x			x								x			x						
Document concomitant medication/therapies	x	x			x								x			x						

a: blood withdrawal may be done at screening or immediately before cycle 1 day 1
b: blood withdrawal to be performed after cycle 2 week 3 and before surgery
c: blood withdrawal to be performed before cycle 3 day 1
d: blood withdrawal to be performed approximately 12 weeks after surgery
e: Cycle 3 day 1 should ideally start within 4-8 weeks after surgery, at the latest: 12 weeks after surgery
f: CT should be performed after completion of cycle 2 and before surgery
g: Surgery should ideally be performed 7-9 weeks after Cycle 1 day 1, but has to be performed 11 weeks after Cycle 1 day 1

Besides the prognostic value, literature provides some support for a predictive value of CMS for response to systemic treatment. In a retrospective analysis of the NSABP C-07 trial on patients (n=1033) with stage III colon cancer, only CMS2 was associated with benefit from oxaliplatin treatment, patients with CMS4 tumors did not benefit from addition of oxaliplatin treatment [16]. The mesenchymal subtype showed no benefit from 5-FU monotherapy compared to no systemic therapy in a non-randomized retrospective analysis of 222 stage III CRC patients [17].

Although being a promising molecular marker, a solid chemotherapy response assessment per subtype has not been performed and it remains unknown whether the difference in long-term outcome between CMS1-3 and CMS4 originates from differences in prognosis or response to therapy.

This makes it impossible to know whether patients with the poor-prognosis subtype (CMS4) have an impaired survival due to the aggressive nature of the tumor or due to a limited response to chemotherapy. Therefore, it is unknown whether these patients should receive chemotherapy or not. This also holds true for the other subtypes. Although CMS1-3 show superior outcomes to CMS4, it is unknown whether this is due to a favorable tumor biology or due to a substantial response to chemotherapy. We therefore believe that a solid chemotherapy response assessment per subtype is an important and essential step to distinguish between prognosis and prediction, and to incorporate the CMSs in clinical decision-making.

Administering neoadjuvant chemotherapy in the suggested study population was proven safe and feasible in the FOXTROT study [8, 9]. Importantly, the pathological tumor response was evidently associated with recurrence free survival. Patients with a complete response (TRG4) developed no recurrences after 5 years of follow-up, compared to 26% of patients that showed no regression at all (TRG0) [9]. This illustrates that the response to chemotherapy of the primary tumor may indeed be a reliable measurement for chemotherapy efficiency.

The primary endpoint of the proposed study is the pathological tumor response, which will be centrally scored using the TRG by Dworak, a highly reproducible scoring system which is often used and clinical meaningful [11]. Evidently, tumor response monitoring using histology requires invasive procedures. As a secondary endpoint, radiological response will be scored by a central board of radiologists and compared to the pathological tumor response to evaluate this noninvasive technique as a response modality. Both the histological and radiological response will be correlated to RFS and OS to assess their prognostic value.

The proposed neoadjuvant approach requires reliable clinical TNM staging to minimize the risk of overtreating patients with stage I or low risk stage II colon cancer. A meta-analysis analyzing the accuracy of T and N staging on CT imaging showed that T1-2 can be reliably distinguished

from T3-4 (sensitivity 96% and specificity 70%), while nodal involvement is unreliable with a pooled sensitivity and specificity of 78% and 68% respectively [18]. Therefore, only T stage will be used to select patients. Second, only patients with an MSS status will be included which will be determined on the biopsies. Following the latest recommendations of the update of the ESMO guideline to refrain from adjuvant chemotherapy in high-risk stage II MSI colon cancer patients as the possible clinical benefit is too low [19]. This was also seen in the FOxTROT trial, where MSI status was associated with a significantly higher rate of poor/no response (96% vs. 66%, $p<0.0001$) [20]. Using the proposed selection of patients with a MSS cT3-4NxM0 colon tumor, up to 26% of patients is estimated to be overtreated [21, 22].

Results from this study, in which we analyze both the pathological and radiological tumor response per CMS, will lead to improved patient stratification and clearer insight into which patients benefit from chemotherapy. This will allow us to identify the group of patients that receives chemotherapy appropriately and the group of patients that may not benefit from the current treatment regimen. Future studies should focus on whether chemotherapy can be withheld in this patient group or on the development of new therapies to improve patient outcome.

LIST OF ABBREVIATIONS

CAF	Cancer Associated Fibroblast
CAPOX	Capecitabin and Oxaliplatin
CMS	Consensus Molecular Subtype
ctDNA	Circulating Tumor DNA
CytoLym	Cytotoxic Lymphocytes
DCCG	Dutch Colorectal Cancer Group
ESMO	European Society of Medical Oncology
MEC	Medical Ethics Committee
MSI	Microsatellite Instable
MSS	Microsatellite Stable
OS	Overall Survival
PLCRC	Prospective Dutch ColoRectal Cancer cohort
RFS	Recurrence Free Survival
TRG	Tumor Response Grading

DECLARATIONS

Ethics approval and consent to participate

The study has been approved by the medical ethics committee Utrecht (MEC18/712). The medical ethics committee Utrecht belongs to the UMC Utrecht and the Prinses Máxima Center. Reference number SL/rc/19/009541. The study will be conducted according to the principles of the Declaration of Helsinki (10th version, Fortaleza 2013) and in concordance with the Dutch Medical Research Improving Human Subjects Act (WMO) and other applicable guidelines, regulations and acts. Authorships will be defined following the International Committee of Medical Journal Editors guidelines [23].

The patients treating physicians, local investigator or research nurse of the participating hospitals will follow ICH-GCP and other applicable regulations in informing the patient and obtaining consent. This includes explaining the CONNECTION-II study to the patient, providing him/her with information such as the expected efficacy and possible side effects, and that refusal to participate will not influence further options for therapy. Before informed consent may be obtained, the investigator should provide the patient ample time and opportunity to inquire about details of the trial and to decide whether or not to participate in the trial. All questions about the trial should be answered to the satisfaction of the patient. Only after written informed consent, the patient will be included in this study. The inclusion has to take place shortly after diagnosis to prevent delay in treatment.

Patients are well informed that participation in voluntary and that they may withdraw at any point during the study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the principal investigator on reasonable request. Results will be communicated via PLCRC, presentations at international conferences and via publications in peer reviewed journals.

Competing interests

The authors declare that they have no competing interests.

Funding

The CONNECTION II trial is funded by the Dutch Cancer Society, Alpe d'HuZes. The Dutch Cancer Society is a non-profit society that funds cancer research and has had no direct influence in the structuring of the trial and will also not benefit financially from the outcome.

REFERENCES

1. Nederlandse Kankerregistratie (NKR), IKNL.
2. Guinney J, Dienstmann R, Wang X, et al. The consensus molecular subtypes of colorectal cancer. *Nat Med* 2015; 21(11):1350-6.
3. van Hagen P, Hulshof MC, van Lanschot JJ, et al. Preoperative chemoradiotherapy for esophageal or junctional cancer. *N Engl J Med* 2012; 366(22):2074-84.
4. Bosset JF, Mercier M, Triboulet JP, et al. Surgical resection with and without chemotherapy in oesophageal cancer. *Lancet* 2002; 360(9340):1173-4; author reply 1175.
5. Cunningham D, Allum WH, Stenning SP, et al. Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. *N Engl J Med* 2006; 355(1):11-20.
6. Sebag-Montefiore D, Stephens RJ, Steele R, et al. Preoperative radiotherapy versus selective postoperative chemoradiotherapy in patients with rectal cancer (MRC CR07 and NCIC-CTG C016): a multicentre, randomised trial. *Lancet* 2009; 373(9666):811-20.
7. Roh MS, Yothers GA, Connell MJO, et al. The impact of capecitabine and oxaliplatin in the preoperative multimodality treatment in patients with carcinoma of the rectum: NSABP R-04. *Journal of Clinical Oncology* 2011; 29(15_suppl):3503-3503.
8. Foxtrot Collaborative G. Feasibility of preoperative chemotherapy for locally advanced, operable colon cancer: the pilot phase of a randomised controlled trial. *Lancet Oncol* 2012; 13(11):1152-60.
9. Matthew T. Seymour DM, and on behalf of the International FOxTROT Trial Investigators. FOxTROT: an international randomised controlled trial in 1052 patients (pts) evaluating neo-adjuvant chemotherapy (NAC) for colon cancer. Journal of Clinical Oncology 2019.
10. Coebergh van den Braak RRJ, van Rijssen LB, van Kleef JJ, et al. Nationwide comprehensive gastro-intestinal cancer cohorts: the 3P initiative. *Acta Oncol* 2018; 57(2):195-202.
11. Dworak O, Keilholz L, Hoffmann A. Pathological features of rectal cancer after preoperative radiochemotherapy. *Int J Colorectal Dis* 1997; 12(1):19-23.
12. Lee J, Sohn I, Do IG, et al. Nanostring-based multigene assay to predict recurrence for gastric cancer patients after surgery. *PLoS One* 2014; 9(3):e90133.
13. Nielsen T, Wallden B, Schaper C, et al. Analytical validation of the PAM50-based Prosigna Breast Cancer Prognostic Gene Signature Assay and nCounter Analysis System using formalin-fixed paraffin-embedded breast tumor specimens. *BMC Cancer* 2014; 14:177.
14. Veldman-Jones MH, Brant R, Rooney C, et al. Evaluating Robustness and Sensitivity of the NanoString Technologies nCounter Platform to Enable Multiplexed Gene Expression Analysis of Clinical Samples. *Cancer Res* 2015; 75(13):2587-93.
15. Weissenberg E. TNM staging of colorectal carcinoma (AJCC 8th edition). *Journal* [serial online]. 2019.
16. Song N, Pogue-Geile KL, Gavin PG, et al. Clinical Outcome From Oxaliplatin Treatment in Stage II/III Colon Cancer According to Intrinsic Subtypes: Secondary Analysis of NSABP C-07/NRG Oncology Randomized Clinical Trial. *JAMA Oncol* 2016; 2(9):1162-9.
17. Roepman P, Schlicker A, Tabernero J, et al. Colorectal cancer intrinsic subtypes predict chemotherapy benefit, deficient mismatch repair and epithelial-to-mesenchymal transition. *Int J Cancer* 2014; 134(3):552-62.
18. Nerad E, Lahaye MJ, Maas M, et al. Diagnostic Accuracy of CT for Local Staging of Colon Cancer: A Systematic Review and Meta-Analysis. *AJR Am J Roentgenol* 2016; 207(5):984-995.

19. Committee EG. eUpdate - Early Colon Cancer Treatment Recommendations. 2019.
20. Murakami1 K, West1 N, Westwood1 A, et al. The Relationship Between DNA Mismatch Repair and Response to FOLFOX-based Pre-operative Chemotherapy in the International Phase III FOxTROT Trial. *Journal of pathology, understanding diseases* 2019; 249.
21. Norgaard A, Dam C, Jakobsen A, et al. Selection of colon cancer patients for neoadjuvant chemotherapy by preoperative CT scan. *Scand J Gastroenterol* 2014; 49(2):202-8.
22. Sjovall A, Blomqvist L, Egenvall M, et al. Accuracy of preoperative T and N staging in colon cancer--a national population-based study. *Colorectal Dis* 2016; 18(1):73-9.
23. ICoMJ E. Defining the role of authors and contributors. *In* Editors ICoMJ, ed.

Chapter 10

General Discussion & Future Perspectives

GENERAL DISCUSSION & FUTURE PERSPECTIVES

Daily practice

With the availability of the MATCH data, we were able to analyze daily practice regarding treatment of colon cancer in a well-defined population and analyze factors associated with poor prognosis and response to treatment. The second chapter gives an overview of the evidence from previously performed and currently ongoing randomized controlled trials (RCTs) regarding systemic treatment in stage II-III colon cancer patients. Results from these studies provide clear evidence for which chemotherapy regimens provide the most optimal patient outcomes. Based on the results from these RCTs, current guidelines recommend adjuvant chemotherapy for patients with high risk stage II and stage III colon cancer. The adjuvant chemotherapy regimen consists of a fluoropyrimidine, either in combination with oxaliplatin, or as monotherapy [1-6]. However, there are various reasons why the results of RCTs may not apply directly to daily clinical practice. For one, due to strict inclusion and exclusion criteria and close monitoring of the safety of participants and their adherence to treatment, ideal conditions are created. However, in daily practice, physicians also have to deal with older, fragile patients for whom standard treatment may not be suitable. Observational studies, in contrast, are based on so called real-world data, such as those from the MATCH study and electronic health records, and often better represent daily practice. The studies performed in this thesis are based on observational data which indeed demonstrates that daily practice regarding treatment is often different from what is recommended in evidence based guidelines. This thesis gives a reflection of actual daily practice regarding the treatment and outcomes of patients with stage I-III colon cancer. Specifically, adjuvant chemotherapy administration seems to vary significantly between patients which is concurrent with previous literature [7-11].

Furthermore, our results show that there are several factors underlying the variation in treatment and patient outcomes. Differences exist in administration of adjuvant treatment between hospitals, but also age and socioeconomic status are associated with differences in treatment. Besides these variables, other factors may be associated with reduced odds of receiving adjuvant chemotherapy. Unfortunately, as described in the fifth chapter, generally the reasons for such are poorly documented. While non-receipt of adjuvant therapy may be entirely appropriate given the circumstances of individual patients, documentation of these considerations is essential for research and advancement of clinical daily practice. Especially because not administering adjuvant chemotherapy can lead to a significant recurrence free survival difference, most present in patients with high risk tumors. The poor documentation on treatment decisions also highlights one of the most important limitations of observational studies. Although observational studies are important to give an accurate reflection of daily practice regarding treatment and outcome, the quality of electronic health records data tends

to be inferior compared with those collected in RCTs. Furthermore, due to the absence of randomization in observational studies, results are more prone to bias due to incomparability of treatment groups and selective dropout. These are limitations of our studies [12]. Therefore, to implement results from RCTs and observational studies in daily practice, it is paramount to critically analyze their results and to place them in perspective regarding their methodology.

Efforts should be aimed to improve documentation on reasons to either administer adjuvant chemotherapy or to refrain from it. A more detailed documentation may help to give further insight in the relevance and feasibility to adhere to the guidelines. Synoptic reporting, which is used in other cancer specialties, could be implemented in oncology as well. There are several examples on how to improve documentation. For instance, a group of ASCO members developed the "Chemotherapy Treatment Summary: A Two-Step Process" which helps to clearly document and summarize the individual treatment [13]. Furthermore, as mentioned in the conclusion of the second chapter, the only way to truly move forward and improve patient care is by joint initiatives and collaborations. In 2009, the nationwide Dutch Colorectal Audit (DCRA) was initiated by Association of Surgeons of the Netherlands (ASN) to monitor, evaluate and improve colorectal cancer (CRC) care. The DCRA is currently widely used as a blueprint for the initiation of other audits, coordinated by the Dutch Institute for Clinical Auditing (DICA). Within 2 years following initiation of the DCRA, all Dutch hospitals participated in the audit. Plus, within three years, guideline compliance for diagnostics, preoperative multidisciplinary meetings and standardized reporting increased; complication-, re-intervention and postoperative mortality rates decreased significantly [14]. Another example of an effective collaboration is the Pathologisch Anatomisch Landelijk Geautomatiseerd Archief (PALGA) and its collaboration with DICA. PALGA is a nationwide histopathology and cytopathology network and archive, encompassing all sixty-four pathology laboratories in The Netherlands [15]. Excerpts of all histopathology and cytopathology reports are generated automatically at the participating laboratories and transferred to the central databank. All excerpts are continuously available to all participating pathology laboratories. The PALGA Foundation and DICA have established a collaboration, so that pathology data can now easily be transferred to the DCRA registration. This increases the quality of the pathology data in the registration, reduces the registration burden within hospitals and thus promotes quality of care. The medical oncology is still lagging behind regarding registration and documentation, although initiatives are being undertaken. The National Health Care Institute in the Netherlands is starting practical tests on the registration of the effects of drug treatment [16]. These case studies provide insight into how healthcare providers can effectively record the effects of drug treatment in the future. Not only the treatment-effect on the disease, but also the influence on the quality of life for patients. One of these case studies is in collaboration with the Prospectief Landelijk ColoRectaal Carcinoom cohort (PLCRC) of the Dutch Colorectal Cancer Group (DCCG) and the Netherlands Comprehensive Cancer Organisation (IKNL) [17-19]. PLCRC collects data from patients with stage I-IV

CRC in the Netherlands and the main goal of the IKNL is to reduce the impact of cancer, with the Netherlands Cancer Registry (NCR) as its core activity. Although this case study focusses on drug treatment for metastatic CRC, the results can also improve the quality of registration and documentation of treatment for non-metastatic colon cancer. Good quality registration makes it possible to assess the value of drug treatment in clinical practice. Another example of a collaboration on improving cancer care is the Taskforce Oncology. The Taskforce Oncology consists of the Dutch Federation of University Medical Centers (NFU), the Dutch Association of Hospitals (NVZ), the Dutch Federation of Cancer Patient Organizations (NFK), the Dutch College of General Practitioners (NHG), platform Oncology - SONCOS of the Federation Medical Specialists, IKNL, Nurses & Caregivers in the Netherlands (V&VN) and the Dutch national Citrien program 'Towards regional oncology networks', who are working together to further improve oncological care. The Taskforce oncology came up with an initiative called the MDO 2.0 project [20, 21]. With this project they aimed to answer the question on how treatment for cancer patients can be discussed in a multidisciplinary way with the right disciplines involved. They found that the solution lies in echeloning the multidisciplinary consultation (MDO): adjusting the level of the MDO to the complexity of the care demanded. This results in different levels of MDO in daily clinical practice, based on the complexity of the patient: local consultation for simple cases, and a regional or national MDO for more complex situations. All of these efforts and successes are the perfect example of effective collaborations with the aim to promote personalized medicine in daily practice.

Another key aspect in quality cancer care is shared decision making. Patients and physicians must jointly decide on a treatment plan in which the personal costs and benefits are weighed up and discussed. Multiple studies, including the ones described in this thesis, have shown that adjuvant chemotherapy can have a significant effect on survival. However, chemotherapy also influence quality of life. The improvement in survival caused by chemotherapy may not outweigh the possible reduction in quality of life for some patients. To optimize shared decision-making and define the best treatment for patients with colon cancer, a better understanding of patients' preferences for treatment is needed. Patients' views, beliefs and priorities can differ substantially from those of their treating physicians [22-24]. These differences in preferences suggest that there is the potential for improvement in patients' well-being. Factors that influence patients' preferences, and trade-offs that patients are willing to make in their choice between treatment can be studies by discrete choice experiments. Results from a study performed in esophageal cancer patients, showed patients are willing to trade off substantial 5-year survival to achieve a reduction in the risk that surgery is necessary[25]. A similar study could be performed on adjuvant chemotherapy in colon cancer patients. Analyzing factors that influence patients' preferences, and trade-offs that they are willing to make in their choice between surgery and surgery followed by adjuvant chemotherapy can optimize personalized medicine. Initiating or enhancing discussions about patient tolerance for toxicities, may help prescribe treatments that entail more appropriate benefit–risk tradeoffs.

Molecular biology

Common mutations, chromosomal changes and translocations can have an effect on important pathways cancer progression. Specific genes can be used as prognostic, and some even predictive, markers for patient outcome [26]. In addition to these gene mutations, alterations in non-coding RNAs, such as circRNAs, can also contribute to carcinogenesis. Since circRNAs have been proven to remain stable in saliva, blood, and exosomes, they make promising biomarkers for the diagnosis, prognosis, and therapeutic assessment of cancer patients. In our studies on daily practice, we did not include biological tumor characteristics in the survival analyses even though several molecular tumor characteristics have a proven effect on patient prognosis. Results from this thesis clearly demonstrate that differences in molecular tumor biology influence patients' prognosis. A deeper understanding of tumor biology and its relation to patient outcomes is of great importance. The results from this thesis provide some insight in the biological behavior of different colon cancer subtypes. In the future, treatment for colon cancer patients will likely be subtype specific by targeting characteristically overexpressed molecular targets per consensus subgroup [27]. Plus, future studies should focus on whether chemotherapy can be withheld in patient groups with specific colon cancer subtypes and on the development of new therapies to improve patient outcome. As previously stated in this thesis, the gold-standard classification strategy relies on genome-wide RNA expression data from sufficient quantities of fresh-frozen bulk tumor, which hampers widespread implementation. Therefore, before CMS can actually be applied in a clinical setting, a more practical, minimally-invasive test to distinguish between subtypes is needed. Our developed methylation marker panel can enable the development of a qPCR DNA methylation assay for distinguishing CMS in patients with CRC. However, a limitation of this marker panel is that it only differentiated between CMS 2 and 3. Currently, no therapies stratifying CMS2 or 3 specifically, have been developed yet, whereas CMS1 and 4 are clinically relevant with respect to survival and treatment. Although other tests are already available for identifying CMS 1 and 4, one test to distinguish between all four would obviously be ideal. Practical and affordable tests to determine CMS will greatly aid in establishing the clinical value of these molecular subtypes as these will enable routine determination of CMS in ongoing CRC research.

Epigenetic gene silencing is one causative factor of colon cancer development, with DNA methylation as major driving force. Global genomic hypomethylation and gene promoter hypermethylation, are major epigenetic changes in colon cancer. We have demonstrated that largely different alternative splicing events are rather common in colon cancer. Therefore, it would be interesting to investigate whether the aberrant expression of circRNAs observed in colon cancer could be, at least partly, attributed to epigenetic changes in the genomic locus from which these circRNAs are produced.

Prediction versus prognosis

The second chapter describes the widespread search for new tumor biology driven therapeutics that has evolved in recent years. This raises the need for corresponding prognostic and predictive biomarkers in order to improve outcome by better patient selection for these different treatments. As described in the introduction of this thesis, biomarkers can be separated in two groups: prognostic biomarkers and predictive biomarkers. In turn, two types of predictive markers can be distinguished: upfront and early predictive markers. The first can be used for patient selection for treatment and the second provides information early on during therapy [28]. Two of the most important developments in recent years regarding biomarkers are ctDNA and CMS. With the introduction of ctDNA, patients with a high risk of recurrence can be identified, proving its prognostic value. Several studies have reported that serial monitoring of ctDNA can also provide valuable information on the efficacy of adjuvant chemotherapy, proving its early predictive value [29-31]. Although ctDNA can function as a prognostic biomarker and early predictive marker, upfront prediction for treatment response is not possible with ctDNA. A high risk of recurrence is not necessarily solved by administering more or heavier chemotherapy regimens. Therefore, predicting risk of recurrence should go hand in hand with upfront prediction of response to chemotherapy. The CMS subtypes have been developed to stratify colon cancer into biologically and clinically distinct subtypes [32]. Literature provides support for a prognostic and predictive value for response to systemic treatment [33, 34]. The CONNECTION-II trial, combines ctDNA and CMS and can provide the results necessary to proceed to future studies in which (neo)adjuvant chemotherapy may be withheld in patients with a specific CMS subtype, who show no benefit from chemotherapy and for whom possible new treatments can be investigated.

Another promising prognostic biomarker with possible predictive value as well, is the Immunoscore [35]. This stratification in colon cancer patients relies on tumor-infiltrating lymphocytes (TILs). Tumor-infiltrating lymphocyte densities have shown prognostic significance in well-defined cohorts of patients with stage II and III colon cancer, and the developed Immunoscore has been externally validated [35-38]. Analysis of TILs could enable more selective use of adjuvant therapy in both stage II and stage III patients [39]. Literature provides some support for a possible predictive potential of TILs for response to chemotherapy but future studies are warranted to analyze the predictive value [38, 40].

Neoadjuvant treatment

Results of the first phase III RCT on neoadjuvant chemotherapy are very promising and are likely to have a big impact on treatment strategy for a large group of high risk stage II/stage III colon cancer patients. The FOXTROT study and the PRODIGE 22 study both showed that

neoadjuvant chemotherapy was well tolerated and with no increase in surgical morbidity [41]. However, this neoadjuvant approach requires reliable clinical TNM staging to minimize the risk of overtreating patients with stage I or low risk stage II colon cancer. In the FOXTROT-study and PRODIGE 22, approximately 25% of patients were erroneously staged and consequently treated with chemotherapy which they would not have been in an adjuvant setting. These numbers are similar to results from previous studies, underlining the need for improving pre-treatment disease assessment in this setting [41-43]. Therefore, improvements are essential to optimize patient selection for neoadjuvant treatment. A recent study evaluated whether staging of colon cancer on CT can be improved in radiologist trainees. Results show that the diagnostic performance of T staging of colon cancer on CT-scan improved significantly after analyzing multiple scans. However, N staging on CT did not show a significant improvement over time. Feedback from an expert in between scans did not lead to an improvement in performance suggesting that experience itself would be sufficient for radiologists to become accurate. This still leaves the need for accurate N staging. Exploratory data suggests a possible role for MRI or PET in preoperative staging [44, 45]. However these techniques have not been validated in randomized trials and data is limited to small case series. More refinement of imaging techniques are needed to optimize patient selection for neoadjuvant treatment.

In conclusion, knowledge on prognosis, prediction and treatment has dramatically improved in the last decade. However, upfront prediction of response to therapy and thereby accurate patient selection for adjuvant chemotherapy is still far from optimal. This thesis shows that the results from real-world data are considerably different than those from RCTs regarding patient selection for treatment, treatment decisions, patient preferences and patient outcomes. We know that fewer than 1 in 20 cancer patients enroll in RCTs [46]. This leaves over 95% of patients for whom the best course of treatment needs to be studied and determined. To accomplish this and thereby practice personalized medicine for each patient, we need to be able to learn from every individual and learn from both multidisciplinary clinical, and molecular data. The MATCH study was the first step towards achieving this goal by including all stage I-III CRC patients within the larger region of Rotterdam and collecting both clinical data and tumor tissue. The MATCH study, together with similar initiatives, continued towards the set-up of the national collaboration PLCRC. In PLCRC, extensive longitudinal clinical data is collected since 2013, together with blood, (tumor) tissue, and repeated patient-reported outcomes (PROs) in patients with stage I-IV CRC that are prospectively followed from primary diagnosis until death. PLCRC approaches to represent the Dutch CRC population and will ultimately meet the current demand for high-quality real-world data [19]. PLCRC, in turn, led to the set-up of the CONNECTION-II trial. The results from CONNECTION-II trial will lead to improved patient stratification and thereby, we hope to finally be able to answer the question raised at the beginning and throughout this thesis: which patients benefit from chemotherapy and which patients are overtreated. Results from the CONNECTION-II trial enables future studies to focus

on whether chemotherapy can be withheld in certain patient groups and on the development of new therapies to improve patient outcome. It is paramount to be able to better predict the risk of recurrence and need for adjuvant chemotherapy per individual patient, and study new treatment approaches for specific subgroups to improve survival but also to avoid overtreatment in the future. The only way to truly move forward and accomplish precision medicine is by initiatives and collaborations such as the MATCH, PLCRC and CONNECTION.

Joining hands and MATCHing patients with their most optimal treatment are the cornerstones in the current era of personalized medicine.

REFERENCES

1. André T, Boni C, Navarro M, et al. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. *J Clin Oncol* 2009; 27(19):3109-3116.
2. Haller DG, Tabernero J, Maroun J, et al. Capecitabine plus oxaliplatin compared with fluorouracil and folinic acid as adjuvant therapy for stage III colon cancer. *J Clin Oncol* 2011; 29(11):1465-1471.
3. Moertel CG, Fleming TR, Macdonald JS, et al. Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. *N Engl J Med* 1990; 322(6):352-8.
4. Kuebler JP, Wieand HS, O'Connell MJ, et al. Oxaliplatin combined with weekly bolus fluorouracil and leucovorin as surgical adjuvant chemotherapy for stage II and III colon cancer: results from NSABP C-07. *J Clin Oncol* 2007; 25(16):2198-204.
5. Yothers G, O'Connell MJ, Allegra CJ, et al. Oxaliplatin as adjuvant therapy for colon cancer: updated results of NSABP C-07 trial, including survival and subset analyses. *J Clin Oncol* 2011; 29(28):3768-74.
6. Andre T, Boni C, Mounedji-Boudiaf L, et al. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. *N Engl J Med* 2004; 350(23):2343-51.
7. van Steenbergen LN, Rutten HJ, Creemers GJ, et al. Large age and hospital-dependent variation in administration of adjuvant chemotherapy for stage III colon cancer in southern Netherlands. *Ann Oncol* 2010; 21(6):1273-8.
8. Rayson D, Urquhart R, Cox M, et al. Adherence to clinical practice guidelines for adjuvant chemotherapy for colorectal cancer in a Canadian province: a population-based analysis. *J Oncol Pract* 2012; 8(4):253-9.
9. Renouf D, Kennecke H, Gill S. Trends in chemotherapy utilization for colorectal cancer. *Clin Colorectal Cancer* 2008; 7(6):386-9.
10. Cree M, Tonita J, Turner D, et al. Comparison of treatment received versus long-standing guidelines for stage III colon and stage II/III rectal cancer patients diagnosed in Alberta, Saskatchewan, and Manitoba in 2004. *Clin Colorectal Cancer* 2009; 8(3):141-5.
11. Keikes L, van Oijen MGH, Lemmens V, et al. Evaluation of Guideline Adherence in Colorectal Cancer Treatment in The Netherlands: A Survey Among Medical Oncologists by the Dutch Colorectal Cancer Group. *Clin Colorectal Cancer* 2018; 17(1):58-64.
12. Collins R, Bowman L, Landray M, et al. The Magic of Randomization versus the Myth of Real-World Evidence. *N Engl J Med* 2020; 382(7):674-678.
13. Developing the medical oncology treatment plan and summary. *J Oncol Pract* 2006; 2(2):95-6.
14. Van Leersum NJ, Snijders HS, Henneman D, et al. The Dutch surgical colorectal audit. *Eur J Surg Oncol* 2013; 39(10):1063-70.
15. Casparie M, Tiebosch AT, Burger G, et al. Pathology databanking and biobanking in The Netherlands, a central role for PALGA, the nationwide histopathology and cytopathology data network and archive. *Cell Oncol* 2007; 29(1):19-24.
16. Nederland Z. Regie op Registers voor Dure Geneesmiddelen gaat testen in de praktijk. 2021.
17. Derksen JWG, Vink GR, Elferink MAG, et al. The Prospective Dutch Colorectal Cancer (PLCRC) cohort: real-world data facilitating research and clinical care. *Sci Rep* 2021; 11(1):3923.
18. Nederlandse Kankerregistratie (NKR), IKNL.

19. Burbach JP, Kurk SA, Coebergh van den Braak RR, et al. Prospective Dutch colorectal cancer cohort: an infrastructure for long-term observational, prognostic, predictive and (randomized) intervention research. *Acta Oncol* 2016; 55(11):1273-1280.
20. oncologie.nu. Echelonnering van het MDO. *Journal* [serial online]. 2018. Accessed May 2021.
21. Vermeer K. MDO 2.0: nieuwe organisatie van MDO's. *Journal* [serial online]. 2019; 9, 2019. Available from: MedNed
22. Chewning B, Bylund CL, Shah B, et al. Patient preferences for shared decisions: a systematic review. *Patient Educ Couns* 2012; 86(1):9-18.
23. Godolphin W. The role of risk communication in shared decision making. *BMJ* 2003; 327(7417):692-3.
24. Thrumurthy SG, Morris JJ, Mughal MM, et al. Discrete-choice preference comparison between patients and doctors for the surgical management of oesophagogastric cancer. *Br J Surg* 2011; 98(8):1124-31; discussion 1132.
25. Noordman BJ, de Bekker-Grob EW, Coene P, et al. Patients' preferences for treatment after neoadjuvant chemoradiotherapy for oesophageal cancer. *Br J Surg* 2018; 105(12):1630-1638.
26. Marmol I, Sanchez-de-Diego C, Pradilla Dieste A, et al. Colorectal Carcinoma: A General Overview and Future Perspectives in Colorectal Cancer. *Int J Mol Sci* 2017; 18(1).
27. Thanki K, Nicholls ME, Gajjar A, et al. Consensus Molecular Subtypes of Colorectal Cancer and their Clinical Implications. *Int Biol Biomed J* 2017; 3(3):105-111.
28. Oldenhuis CN, Oosting SF, Gietema JA, et al. Prognostic versus predictive value of biomarkers in oncology. *Eur J Cancer* 2008; 44(7):946-53.
29. Reinert T, Henriksen TV, Christensen E, et al. Analysis of Plasma Cell-Free DNA by Ultradeep Sequencing in Patients With Stages I to III Colorectal Cancer. *JAMA Oncol* 2019; 5(8):1124-1131.
30. Tarazona N, Gimeno-Valiente F, Gambardella V, et al. Targeted next-generation sequencing of circulating-tumor DNA for tracking minimal residual disease in localized colon cancer. *Ann Oncol* 2019; 30(11):1804-1812.
31. Tie J, Vogelstein B, Gibbs P. Circulating Tumor DNA as a Prognostic Marker in Stage III Colon Cancer-Reply. *JAMA Oncol* 2020; 6(6):932-933.
32. Guinney J, Dienstmann R, Wang X, et al. The consensus molecular subtypes of colorectal cancer. *Nat Med* 2015; 21(11):1350-6.
33. Song N, Pogue-Geile KL, Gavin PG, et al. Clinical Outcome From Oxaliplatin Treatment in Stage II/III Colon Cancer According to Intrinsic Subtypes: Secondary Analysis of NSABP C-07/NRG Oncology Randomized Clinical Trial. *JAMA Oncol* 2016; 2(9):1162-9.
34. Roepman P, Schlicker A, Tabernero J, et al. Colorectal cancer intrinsic subtypes predict chemotherapy benefit, deficient mismatch repair and epithelial-to-mesenchymal transition. *Int J Cancer* 2014; 134(3):552-62.
35. Pages F, Mlecnik B, Marliot F, et al. International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and accuracy study. *Lancet* 2018; 391(10135):2128-2139.
36. Idos GE, Kwok J, Bonthala N, et al. The Prognostic Implications of Tumor Infiltrating Lymphocytes in Colorectal Cancer: A Systematic Review and Meta-Analysis. *Scientific Reports* 2020; 10(1):3360.
37. Sinicrope FA, Shi Q, Hermitte F, et al. Contribution of Immunoscore and Molecular Features to Survival Prediction in Stage III Colon Cancer. *JNCI Cancer Spectrum* 2020; 4(3).

38. Bernhard M, Carlo B, Gabriela B, et al. Multicenter International Society for Immunotherapy of Cancer Study of the Consensus Immunoscore for the Prediction of Survival and Response to Chemotherapy in Stage III Colon Cancer. *Journal of Clinical Oncology* 2020; 38(31):3638-3651.
39. Sinicrope FA, Graham RP. Tumor-Infiltrating Lymphocytes for Prognostic Stratification in Nonmetastatic Colon Cancer-Are We There Yet? *JAMA Oncol* 2021.
40. Pages F, Andre T, Taieb J, et al. Prognostic and predictive value of the Immunoscore in stage III colon cancer patients treated with oxaliplatin in the prospective IDEA France PRODIGE-GERCOR cohort study. *Ann Oncol* 2020; 31(7):921-929.
41. Karoui M, Rullier A, Piessen G, et al. Perioperative FOLFOX 4 Versus FOLFOX 4 Plus Cetuximab Versus Immediate Surgery for High-Risk Stage II and III Colon Cancers: A Phase II Multicenter Randomized Controlled Trial (PRODIGE 22). *Ann Surg* 2020; 271(4):637-645.
42. Norgaard A, Dam C, Jakobsen A, et al. Selection of colon cancer patients for neoadjuvant chemotherapy by preoperative CT scan. *Scand J Gastroenterol* 2014; 49(2):202-8.
43. Sjovall A, Blomqvist L, Egenvall M, et al. Accuracy of preoperative T and N staging in colon cancer--a national population-based study. *Colorectal Dis* 2016; 18(1):73-9.
44. Tsunoda Y, Ito M, Fujii H, et al. Preoperative diagnosis of lymph node metastases of colorectal cancer by FDG-PET/CT. *Jpn J Clin Oncol* 2008; 38(5):347-53.
45. Park SY, Cho SH, Lee MA, et al. Diagnostic performance of MRI- versus MDCT-categorized T3cd/T4 for identifying high-risk stage II or stage III colon cancers: a pilot study. *Abdom Radiol (NY)* 2019; 44(5):1675-1685.
46. Unger JM, Cook E, Tai E, et al. The Role of Clinical Trial Participation in Cancer Research: Barriers, Evidence, and Strategies. *Am Soc Clin Oncol Educ Book* 2016; 35:185-98.

Chapter 11

Summary

SUMMARY

In this thesis, we focused on addressing the challenging aspects in daily practice regarding prognosis and treatment for stage I-III colon cancer patients.

Chapter 2 describes a systematic review in which we assessed the current landscape of (neo) adjuvant therapy and patient selection for stage II and III colon cancer. The efficacy of FOLFOX and CAPOX was confirmed in multiple randomized controlled trials (RCTs). The addition of targeted therapies to chemotherapy showed no survival benefit in stage II and III colon cancer patients. Multiple RCTs, that compared different monotherapy schedules of fluoropyrimidines, showed equivalent effect in stage III patients. Results from the IDEA-study showed that 3 months of adjuvant CAPOX was non-inferior to 6 months regarding survival for most stage III patients. In addition, significantly less toxicity was seen due to this shortening in duration. One RCT compared neoadjuvant chemotherapy with adjuvant chemotherapy and results showed a reduced risk of 2-year recurrence rate in the perioperative chemotherapy group. Neoadjuvant immunotherapy has shown promising results with the potential to become the new standard of care for a specific patient group. From this study, we conclude that the only significant progress made in systemic treatment the last decade is reducing the duration of adjuvant chemotherapy from 6 to 3 months in stage III patients. None of the RCTs that studied new systemic therapies showed survival benefit over conventional therapies. Knowledge about prognosis and prediction has improved by molecular analyses, however, validation in RCTs regarding the added value of biomarkers has not yet been achieved. It is essential that promising developments such as neoadjuvant treatment, immunotherapy for MSI and ctDNA are studied in RCTs followed by validation to be able to implement these in daily practice. Given the increasing number of CC subgroups resulting in smaller patient numbers, international collaborations such as the IDEA and FOxTROT are paramount.

In **chapter 3**, we characterized a cohort of patients who survived up to five years without recurrence and identified factors predicting the probability of long-term recurrence free survival. Analysis of data of 754 patients revealed a 5-year recurrence-free survival rate of 65%. Patients with stage I disease, T1- and N0-tumor had the highest probability of cure (94%, 95% and 90%, respectively). Those with a T4-tumor or N2-tumor had the lowest probability of cure (62% and 50%, respectively). A peak in the mortality rate for older patients early in follow-up suggests early excess mortality as an explanation. A similar trend was observed for patients with stage III disease, poor tumor grade, postoperative complications, sarcopenia and R1 resections. From this study, we conclude that even though most patients will be cured from CRC with standard therapy, standard therapy is insufficient for those with poor prognostic factors, such as high T- and N-stage and poor differentiation grade.

Chapter 4 describes the daily practice regarding treatment and patient outcomes across seven hospitals. We analyzed differences between the hospitals by performing mixed effects Cox proportional hazards models. Data of 1,747 patients diagnosed with stage I-III colon cancer were included. Independent predictors for impaired recurrence free survival (RFS) included T-stage and incomplete resection margins. Adjuvant chemotherapy was associated with longer RFS. Between-hospital differences are present regarding RFS. Independent predictors for impaired overall survival (OS) included male gender, older age, ASA-score III/IV and lung disease(s). Colon descendens tumors and adjuvant chemotherapy were associated with longer OS. No between-hospitals differences were found in OS. Administration of adjuvant chemotherapy in stage III patients was significantly different between hospitals which translated in a difference in RFS but not OS, between hospitals in stage III patients. We concluded that variation exists between hospitals in the adjuvant treatment of colon cancer, which, after correcting for case-mix and chance variation, translates in differences regarding RFS but not OS.

Chapter 5 continues assessing daily practice regarding treatment with adjuvant chemotherapy for patients with stage III colon cancer patients. Data of 575 patients were included of whom 61% received adjuvant chemotherapy. In 39% of patients who did not receive adjuvant chemotherapy, no reason was documented. Only age was predictive for receiving chemotherapy. Patients who received adjuvant chemotherapy had longer RFS. High T- and N-stage were associated with poorer RFS. Risk groups were identified with distinct prognosis and treatment effect and a nomogram was built to visualize individualized RFS differences. This study shows considerable variation in guideline adherence to adjuvant chemotherapy and poor documentation on reasons for non-adherence.

In **chapter 6** the association between socioeconomic status (SES) with short and long-term outcome in 965 patients undergoing curative surgery for stage I-III CRC was assessed. Patients with a lower SES more often had diabetes, more often underwent an open surgical procedure, and had more comorbidities. In addition, patients with a lower SES were less likely to receive (neo)adjuvant treatment. Lower SES was associated with an increased risk of postoperative complications and lower cancer-specific survival.

In **chapter 7**, fresh-frozen tumor tissue samples of 181 patients with stage I/II colon cancer, were analyzed. We identified circular RNAs (circRNAs) from RNAseq data, investigated common biology related to circRNA expression, and studied the association between circRNAs and relapse status, tumor stage, consensus molecular subtypes (CMS), tumor localization and microsatellite instability (MSI). We identified 2606 unique circRNAs. 277 circRNAs were repeatedly occurring in at least 20 patients. We validated four circRNAs by Sanger sequencing. We found that circRNAs were often co-expressed and that high diversity in circRNAs was associated with favorable disease-free survival (DFS). Absence of circMGA was significantly

associated with relapse, whereas circSATB2, circNAB1, and circCEP192 were associated with both MSI and CMS. This study represents a showcase of the potential clinical utility of circRNAs for prognostic stratification in patients with stage I-II colon cancer and demonstrated that high diversity in circRNAs is associated with favorable DFS.

In **chapter 8,** fresh-frozen tumor tissue of 239 patients with stage I-III CRC were analysed to identify a panel of methylation markers to distinguish between CMS2 and 3. We found that overall methylation profiles differed between CMS2 and CMS3 tumors. We build two different prediction models and subsequently different marker panels for classification of CMS2 and CMS3 based on 15, 10 or 5 methylation markers and validated our obtained models in data from the Cancer Genome Atlas project. For both panels, even when using only 5 markers, the sensitivity, specificity, and accuracy were >90%.These highly sensitive and specific methylation marker panel can be used to distinguish CMS2 and 3 and enables future development of a qPCR DNA methylation assay in patients with CRC to provide a specific and non-invasive classification tool.

In the final chapter, **chapter 9,** we describe the rationale and protocol of a single arm intervention study. As can be concluded from the previous chapters, patient selection for adjuvant chemotherapy in early stage colon cancer is far from optimal. It has been shown that CMS subtypes have prognostic and/or predictive value. Due to postoperative timing of chemotherapy in current guidelines, tumor response to chemotherapy per CMS is not known, which makes the differentiation between the prognostic and predictive value impossible. Therefore, we propose to assess the tumor response per CMS in the neoadjuvant chemotherapy setting. In this prospective, single arm, multicenter intervention study, 262 patients with resectable microsatellite stable cT3-4NxM0 colon cancer will be treated with two courses of neoadjuvant and two courses of adjuvant capecitabine and oxaliplatin. The primary endpoint is the pathological tumor response to neoadjuvant chemotherapy per CMS. Secondary endpoints are radiological tumor response, the prognostic value of these responses for recurrence free survival and overall survival and the differences in CMS classification of the same tumor before and after neoadjuvant chemotherapy. This study can provide the results necessary to proceed to future studies in which (neo)adjuvant chemotherapy may be withhold in patients with a specific CMS subtype, who show no benefit from chemotherapy and for whom possible new treatments can be investigated.

Chapter 12

Nederlandse Samenvatting

SAMENVATTING

In dit proefschrift hebben we ons gericht op het identificeren en analyseren van gecompliceerde aspecten ten aanzien van de dagelijkse praktijk met betrekking tot prognose en behandeling van patienten met stadium I-III colon carcinoom .

Hoofdstuk 2 beschrijft een systematic review waarin we de huidige praktijk rondom (neo)adjuvante therapie voor patiënten met stadium II en III colon carcinoom hebben onderzocht. De effectiviteit van FOLFOX en CAPOX werd gevalideerd in meerdere randomized controlled trials (RCTs). De toevoeging van doelgerichte therapieën (targeted therapies) aan chemotherapie liet geen overlevingsvoordeel zien bij patiënten met stadium II en III colon carcinoom. Resultaten van meerdere RCT's lieten vergelijkbare resultaten zien tussen verschillende monotherapieschema's met fluoropyrimidines bij stadium III-patiënten. Resultaten van de IDEA-studie toonden aan dat 3 maanden adjuvante CAPOX niet-inferieur was aan 6 maanden ten aanzien van overleving voor de meeste patiënten met stadium III-patiënten. Bovendien werd er door deze verkorting in duur significant minder toxiciteit gezien. Eén RCT vergeleek neoadjuvante chemotherapie met adjuvante chemotherapie en de resultaten toonden een verminderd risico op 2-jaars recidief in de perioperatieve chemotherapiegroep. Neoadjuvante immunotherapie laat veelbelovende resultaten zien wat mogelijk de nieuwe standaard therapie kan worden voor een specifieke patiëntengroep met colon carcinoom. Uit deze studie concluderen we dat de enige significante vooruitgang die de afgelopen tien jaar is geboekt in systemische behandeling, het verminderen van de duur van adjuvante chemotherapie bij stadium III-patiënten is. Geen van de RCT's waarin nieuwe systemische therapieën werden onderzocht toonden overlevingsvoordeel ten opzichte van conventionele therapieën. Kennis over prognose en voorspelling is aanzienlijk verbeterd door moleculaire analyses, maar validatie in RCT's ten aanzien van de toegevoegde waarde van biomarkers is nog niet bereikt. Het is essentieel dat de veelbelovende ontwikkelingen zoals neoadjuvante behandeling, immunotherapie voor MSI, en ctDNA in RCT's worden onderzocht en vervolgens worden gevalideerd om deze daadwerkelijk in de dagelijkse praktijk te kunnen implementeren. Het toenemend aantal subgroepen in colon carcinoom resulteert in kleinere patiënten aantallen. Hierdoor zijn internationale samenwerkingen zoals de IDEA en FOxTROT van essentieel belang om patiënt-selectie en behandeling te verbeteren.

In **hoofdstuk 3** hebben we een cohort van patiënten gekarakteriseerd die tot vijf jaar zonder recidief hebben overleefd en hebben we factoren geïdentificeerd die de kans op genezing voorspellen. Analyses van data van 754 patiënten liet een genezingspercentage van 65% zien. Patiënten met stadium I-ziekte, T1- en N0-tumor hadden de grootste kans op genezing (respectievelijk 94%, 95% en 90%). Patiënten met een T4-tumor of N2-tumor hadden de laagste kans op genezing (respectievelijk 62% en 50%). Een piek in het sterftecijfer voor oudere

patiënten aan het begin van de follow-up suggereert een vroege oversterfte als verklaring. Een vergelijkbare trend werd waargenomen bij patiënten met stadium III-ziekte, slechte tumordifferentiatie, postoperatieve complicaties, sarcopenie en R1-resecties. Het overgrote deel van de patiënten zal dus genezen van colorectaal carcinoom middels standaard behandeling. Echter, uit deze studie concluderen we dat de huidige therapie-strategie onvoldoende effectief is voor mensen met slechte prognostische factoren, waaronder een hoog T- en N-stadium en een slechte differentiatiegraad.

Hoofdstuk 4 beschrijft de dagelijkse praktijk met betrekking tot behandeling en patiëntuitkomsten in zeven ziekenhuizen. We hebben de verschillen tussen de ziekenhuizen geanalyseerd door middel van proportional hazards mixed-effects modellen. Gegevens van 1.747 patiënten met de diagnose stadium I-III colonkanker werden geïncludeerd. Onafhankelijke voorspellers voor slechtere recidiefvrije overleving waren onder meer T-stadium en onvolledige resectiemarges. Behandeling met adjuvante chemotherapie was geassocieerd met langere recidiefvrije overleving. Analyses waarbij we gecorrigeerd hebben voor patiënt-variatie en toeval, laten significante verschillen zien tussen ziekenhuizen met betrekking tot recidiefvrije overleving. Onafhankelijke voorspellers voor slechtere algehele overleving waren mannelijk geslacht, oudere leeftijd, ASA-score III/IV en longziekte(n). Tumoren in het colon descendens en adjuvante chemotherapie waren geassocieerd met langere algehele overleving. Er werden geen significante verschillen tussen ziekenhuizen gevonden in algehele overleving. Behandeling met adjuvante chemotherapie bij stadium III-patiënten was significant verschillend tussen ziekenhuizen. Tevens was er ook in deze groep een significant verschil in recidiefvrije overleving, maar niet in algehele overleving tussen de ziekenhuizen. We concluderen dat er variatie bestaat tussen ziekenhuizen in de adjuvante behandeling van stadium I-III colon carcinoom, die zich, na correctie voor case-mix en toevalsvariatie, vertaalt in significante verschillen met betrekking tot recidiefvrije overleving maar niet in algehele overleving.

Hoofdstuk 5 gaat verder met het beoordelen van de dagelijkse praktijk met betrekking tot de behandeling met adjuvante chemotherapie voor patiënten met stadium III colon carcinoom. Data van 575 patiënten werden geïncludeerd, van wie 61% adjuvante chemotherapie kreeg. Bij 39% van de patiënten die geen adjuvante chemotherapie kregen, werd geen reden gedocumenteerd in de patiëntendossiers. Alleen de leeftijd was voorspellend voor het krijgen van chemotherapie. Patiënten die adjuvante chemotherapie kregen, hadden een significant langere recidiefvrije overleving. Hoge T- en N-stadia waren geassocieerd met slechtere recidiefvrije overleving. Risicogroepen werden geïdentificeerd met een duidelijk verschil in prognose en behandelingseffect van chemotherapie. Een nomogram werd gemaakt om geïndividualiseerde overlevings-verschillen te visualiseren. Deze studie laat aanzienlijke variatie zien in de behandeling met adjuvante chemotherapie en daarbij slechte documentatie over redenen voor het niet geven van deze therapie.

In **hoofdstuk 6** werd de associatie tussen sociaaleconomische status (SES) en resultaten op korte- en lange termijn onderzocht bij 965 patiënten die curatieve chirurgie ondergingen voor stadium I-III colorectale kanker. Patiënten met een lagere SES hadden vaker diabetes, ondergingen vaker een open chirurgische ingreep en hadden meer comorbiditeit. Bovendien werden patiënten met een lagere SES minder vaak behandeld met (neo)adjuvante therapie. Een lagere SES was significant geassocieerd met een verhoogd risico op postoperatieve complicaties en een slechtere kanker specifieke overleving.

Om de invloed van tumorkarakteristieken nader te besturen werd in **hoofdstuk 7** vers ingevroren tumorweefsel van 181 patiënten met stadium I/II colon carcinoom geanalyseerd. We identificeerden circulaire RNA's (circRNA's) uit RNAseq-gegevens. We analyseerde de algemene biologie gerelateerd aan circRNA-expressie en bestudeerden de associatie tussen circRNA's en recidiefstatus, tumorstadium, tumorlokalisatie, consensus moleculaire subtypes (CMS) en MSI. We identificeerden 2606 unieke circRNA's waarvan er 277 herhaaldelijk voorkwamen bij ten minste 20 patiënten. Vier circRNA's zijn gevalideerd door middel van Sanger-sequencing. We ontdekten dat circRNA's vaak samen tot expressie kwamen en dat een hoge diversiteit in circRNA's geassocieerd was met een gunstige ziektevrije overleving. Analyses naar individuele circRNA's toonde aan dat afwezigheid van circMGA significant geassocieerd was met recidief ziekte, terwijl circSATB2, circNAB1 en circCEP192 geassocieerd waren met zowel MSI als CMS. Deze studie laat de potentiële klinische waarde zien van circRNA's voor prognostische stratificatie bij patiënten met stadium I-II colon carcinoom en toont aan dat een hoge diversiteit in circRNA's geassocieerd is met gunstige ziektevrije overleving.

In **hoofdstuk 8** werd vers ingevroren tumorweefsel van 239 patiënten met stadium I-III colorectaal carcinoom geanalyseerd om een panel van methylatiemarkers te identificeren om onderscheid te maken tussen CMS2 en 3. We ontdekten dat de algehele methylatieprofielen verschilden tussen CMS2- en CMS3-tumoren. We bouwden twee verschillende predictiemodellen en vervolgens twee verschillende marker panels voor de classificatie van CMS2 en CMS3 op basis van 15, 10 of 5 methylatiemarkers. Deze modellen hebben we gevalideerd in data van het Cancer Genome Atlas-project. Voor beide markerpanels waren, zelfs wanneer slechts 5 markers werden gebruikt, de sensitiviteit, specificiteit en nauwkeurigheid > 90%. Deze zeer sensitieve en specifieke methylatiemarker panels kunnen worden gebruikt om CMS2 en 3 te onderscheiden en maken toekomstige ontwikkeling mogelijk van een qPCR-DNA-methylatietest in patiënten met colorectaal carcinoom om als specifieke en niet-invasieve classificatietest te gebruiken.

In het laatste hoofdstuk, **hoofdstuk 9,** beschrijven we de rationale en het studieprotocol van een interventiestudie. Zoals uit de voorgaande hoofdstukken kan worden geconcludeerd, is de selectie van patiënten voor adjuvante chemotherapie bij een vroeg stadium van carcinoom

verre van optimaal. Het is aangetoond dat CMS subtypen een prognostische en/of predictieve waarde hebben. Vanwege de postoperatieve timing van chemotherapie in de huidige standaard behandeling, is de tumorrespons op chemotherapie per CMS niet te bepalen. Hierdoor is het onmogelijk om het onderscheid te maken tussen de prognostische en voorspellende waarde van CMS. Daarom hebben we een studie opgezet om de tumorrespons per CMS te beoordelen in de neoadjuvante chemotherapie setting. In deze prospectieve, single-arm, multicenter interventiestudie zullen 262 patiënten met een resecteerbaar, microsatelliet-stabiel cT3-4NxM0 colon carcinoom worden behandeld met twee kuren neoadjuvante en twee kuren adjuvante capecitabine en oxaliplatine. Het primaire eindpunt is de pathologische tumorrespons op neoadjuvante chemotherapie per CMS. Secundaire eindpunten zijn radiologische tumorrespons, de prognostische waarde van deze responsen voor recidiefvrije overleving en algehele overleving en de verschillen in CMS-classificatie van dezelfde tumor voor en na neoadjuvante chemotherapie. Deze studie kan de resultaten opleveren die nodig zijn om toekomstige studies op te zetten waarin (neo)adjuvante chemotherapie achterwege kan blijven bij patiënten met een specifiek CMS-subtype, die geen baat hebben bij chemotherapie en voor wie mogelijke nieuwe behandelingen kunnen worden onderzocht.

Appendices

List of Publications
PhD Portfolio
Acknowledgements
About the author

LIST OF PUBLICATIONS

This thesis

2021 **I. van den Berg**, R.R.J. Coebergh van den Braak, W.M. Bramer, J.N.M. IJzermans, M. Koopman
A Decade of Systemic Treatment for Stage II-III Colon Cancer Patients: What has changed and what's to come? A systematic review of the randomized trials
Submitted

2021 **I. van den Berg,** C.H.M. Maas, R.R.J. Coebergh van den Braak, H.F. Lingsma, J.N.M. IJzermans
Between-hospital variation in treatment and outcomes in stage I-III colon cancer patients
Submitted

2021 **I. van den Berg,** M. Smid, R.R.J. Coebergh van den Braak, M.A van de Wiel, C.H.M. van Deurzen, V. de Weerd, J.W.M. Martens, J.N.M. IJzermans, S.M. Wilting
A panel of DNA methylation markers for the classification of Consensus Molecular Subtypes 2 and 3 in patients with colorectal cancer
Under revision at Molecular Oncology

2021 **I. van den Berg,** M. Smid, R.R.J. Coebergh van den Braak, C.H.M. van Deurzen, V. de Weerd, J.N.M. IJzermans, J.W.M. Martens, S.M. Wilting
Circular RNA in chemonaive lymph node negative colon cancer patients
Cancers 2021 Apr 15;13(8):1903.

2021 **I. van den Berg,** S. van de Weerd, D. van Klaveren, R.R.J. Coebergh van den Braak, J.H.J.M. van Krieken, M. Koopman, J.M.L. Roodhart, J.P. Medema, J.N.M. IJzermans
Daily practice in guideline adherence to adjuvant chemotherapy in stage III colon cancer and predictors of outcome
Eur J Surg Oncol. 2021 Mar 16:S0748-7983(21)00376-0.

2021 **I. van den Berg,** R.R.J. Coebergh van den Braak, J.L.A. van Vugt, J.N.M. IJzermans, S. Buttner
Actual Survival After Resection of Primary Colorectal Cancer, Results from a Prospective Multicenter Study
World J Surg Oncol. 2021 Apr 5;19(1):96.

2020 **I. van den Berg,** S. van de Weerd, J.M.L. Roodhart, G.R. Vink, R.R.J. Coebergh van den Braak, C.R. Jimenez, S.G. Elias, D. van Vliet, M. Koelink, E. Hong, W.M.U. van Grevenstein, M.G.H. van Oijen, R.G.H. Beets-Tan, J.H.J.M. van Krieken, J.N.M. IJzermans, J.P. Medema, M. Koopman and on behalf of the CONNECTION-study group
Improving clinical management of colon cancer through CONNECTION, a nation-wide colon cancer registry and stratification effort (CONNECTION II trial): rationale and protocol of a single arm intervention study.
BMC Cancer. 2020 Aug 18;20(1):776.

2019 **I. van den Berg,** S. Buttner, R.R.J. Coebergh van den Braak, K.H.L. Ultee, H.F. Lingsma, J.L.A. van Vugt, J.N.M. IJzermans
Low Socioeconomic Status is associated with worse outcomes after surgery for colorectal cancer: results from a large, Multicenter study
J Gastrointest Surg. 2020 Nov;24(11):2628-2636.

Other publications

2021 **I. van den Berg,** A.M.J. van Nistelrooij, S.M. Lagarde, B.P.L. Wijnhoven
Risk factors for readmission following esophagectomy and gastrectomy for cancer.
Submitted

2020 C. Kamphues, S. Kadowaki, N. Amini, **I. van den Berg,** J. Wang , N. Andreatos, Y. Sakamoto , T. Ogura, M. Kakuta, A. Pikouli, D. Geka, N. Daitoku, M. Theochari, T. Akiyama, E. Antoniou, E. Pikoulis, G. Theodoropoulos, K. Imai, J.N.M. Ijzermans, G.A. Margonis, K. Akagi, M.E. Kreis
The interplay of KRAS mutational status with tumor laterality in non-metastatic colorectal cancer: An international, multi-institutional study in patients with known KRAS, BRAF, and MSI status.
J Surg Oncol. 2021 Mar;123(4):1005-1014.

2018 **2017 European Society of Coloproctology (ESCP) collaborating group.**
Safety of primary anastomosis following emergency left sided colorectal resection: an international, multi-centre prospective audit
Colorectal Dis. 2018 Sep;20 Suppl 6:47-57.

2018 **2017 European Society of Coloproctology (ESCP) collaborating group.**
Association of mechanical bowel preparation with oral antibiotics and anastomotic leak following left sided colorectal resection: an international, multi-centre, prospective audit
Colorectal Dis. 2018 Sep;20 Suppl 6:15-32.

2018 **2017 European Society of Coloproctology (ESCP) collaborating group.**
An international multicentre prospective audit of elective rectal cancer surgery; operative approach versus outcome, including transanal total mesorectal excision (TaTME)
Colorectal Dis. 2018 Sep;20 Suppl 6:33-46.

2018 **2017 European Society of Coloproctology (ESCP) collaborating group.**
Evaluating the incidence of pathological complete response in current international rectal cancer practice: the barriers to widespread safe deferral of surgery
Colorectal Dis. 2018 Sep;20 Suppl 6:58-68.

2018 **2017 and 2015 European Society of Coloproctology (ESCP) collaborating groups.**
The impact of conversion on the risk of major complication following laparoscopic colonic surgery: an international, multicenter prospective audit
Colorectal Dis. 2018 Sep;20 Suppl 6:58-68.

PHD PORTFOLIO

PhD student	Inge van den Berg
PhD period	2017-2021
Erasmus MC department	Surgery
UMC Utrecht department	Medical oncology
Supervisors	Prof. dr. J.N.M. IJzermans and prof. dr. M. Koopman

Year		Workload ECTS
	Research Skills	
2017	BROK 'Basiscursus Regelgeving Klinisch onderzoek'	1.0
2017	Introduction to Data-analysis	1.0
2018	Research Integrity, Erasmus MC	0.3
2017	Introduction to Data-analysis	1.0
2017	Genomics in Molecular Medicine	1.4
2017	Basic Introduction Course on SPSS	1.0
2018	Survival analysis for Clinicians	1.9
2018	Research Integrity	0.3
2020	Biomedical English writing course	2.0
2021	Photoshop and Illustrator	0.3
2021	Indesign CC	0.15
	Scientific presentations	
2017	NVvH Chirurgendagen	1.0
2017	Erasmus MC Cancer Institute Research Day	2.0
2018	Wetenschapsdag Heelkunde Erasmus MC	2.0
2019	Najaarsdag NVvH	2.0
2019	European Multidisciplinary Colorectal Cancer Congress	1.0
2019	European society of surgical oncology	2.0
2020	NVGE Digestive Disease Days	2.0
2020	Erasmus MC Cancer Institute Research Day	2.0
2021	ASCO: American Society of Clinical Oncology	1.0
	Teaching	
2017-2020	Coachinh bachelor students	1.0
2017-2019	Examination Basic Life Support	1.0
2017-2021	Supervision bachelor student medicine	4.0
Total		**31.25**

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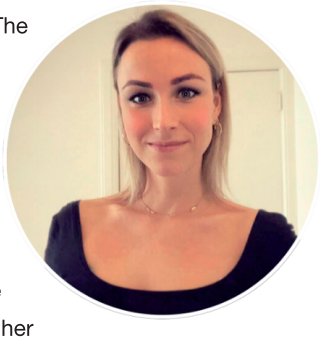
ABOUT THE AUTHOR



Inge van den Berg was born on the 5th of June, 1990 in Delft, The Netherlands. In 2008 she graduated secondary school, Sint Stanislas College, Delft. In 2008 she moved to Groningen to start her medical training at the Rijksuniversiteit Groningen. In 2012 she moved to Rotterdam to continue her medical training at the Erasmus University. During her clinical rotations she did an elective in anesthesiology at the Reinier de Graaf Hospital in Delft and an elective in emergency medicine at the IJsselland Hospital in Capelle aan den Ijssel. She did her senior internship in surgery at the Reinier de Graaf Hospital in Delft.
The first steps in research were made during her master thesis at the surgical department from the OLVG hospital in Amsterdam on totally implantable venous access devices. After obtaining her medical degree in January 2016 she started working as a resident at the department of Surgery of the Ikazia Hospital in Rotterdam where she worked until April 2017 (dr. P.T. Den Hoed). In June 2017 she started working fulltime on her PhD project conducting the research described in this thesis, under supervision of prof. dr. J.N.M. IJzermans (department of Surgery, Erasmus University MC Rotterdam) and prof. dr. M. Koopman (department of Medical Oncology, UMC Utrecht). From September 2021 onwards, Inge is working as a resident in internal medicine at the st. Antonius Ziekenhuis in Nieuwegein (dr. P.Chr. de Jong).