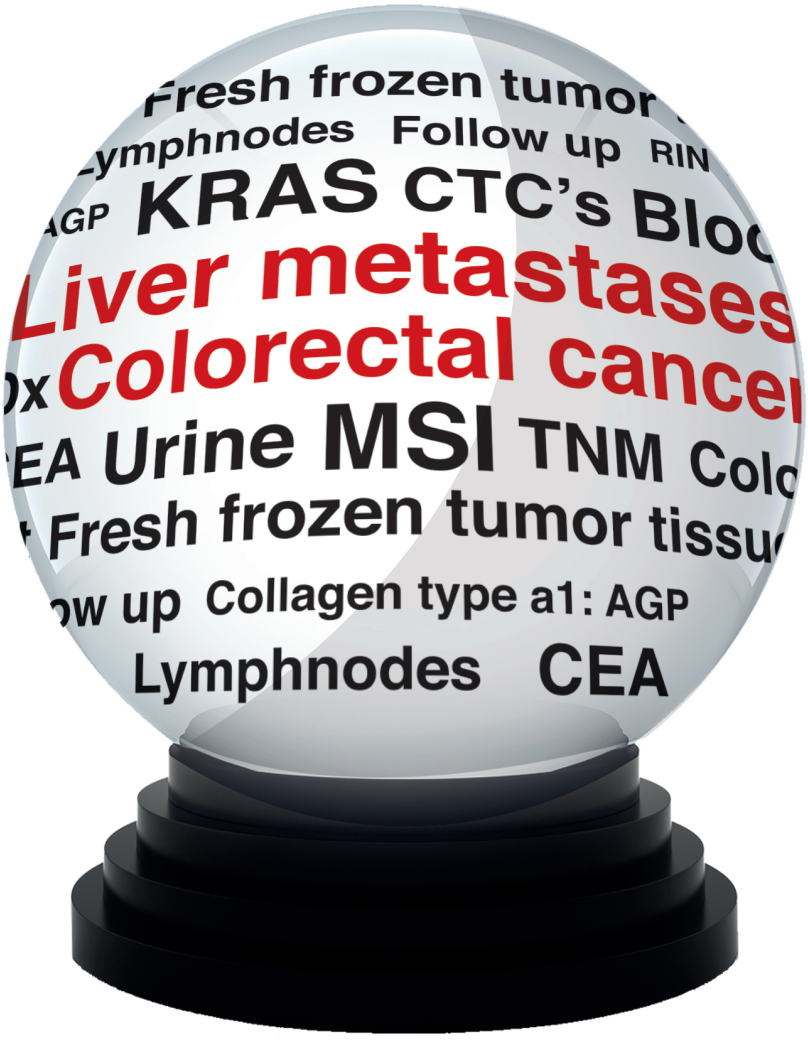

COLORECTALCANCER AND LIVERMETASTASES

DETERMINANTS OF OUTCOME

ZARINA S. LALMAHOMED

Colorectal Cancer and Liver Metastases

Determinants of outcome

Zarina Lalmahomed

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Voor Oom Roy

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Chapter 1

Introduction

Worldwide, colorectal cancer (CRC) is the third most common cancer in men and the second most common cancer among women. Yearly, 1.36 million people are diagnosed with this cancer, and the incidence is still rising. Each year, it is associated with 694,000 deaths from widespread metastatic disease[1]. Surgery alone can cure a large group of CRC patients who present without distant metastases, and even without adjuvant therapy, up to 50% of these patients will remain disease free[2-4]. In spite of optimal surgical treatment, however, between 30–50% of patients with stage II and III CRC will develop metastatic disease[2-4]. Postoperative adjuvant chemotherapy may significantly reduce this risk, but there are some drawbacks. At present, identification of those patients at risk for developing recurrent disease is not possible, and all such patients should be treated, only a selected group of stage II and III patients may benefit from the adjuvant treatment. In addition, the response rate to chemotherapy for individual patients is unknown, and with the lack of predictive markers, many patients may be exposed to the toxicity of chemotherapy without having any benefit, even developing a recurrence in spite of chemo treatment. In patients with stage II CRC, only 15% respond to adjuvant chemotherapy, with an improved survival of less than 5% at 5 years[5-7]. For this reason, the decision to offer adjuvant chemotherapy should be balanced against the possible risks of treatment-related toxicity. Better tools are needed to help clinicians identify the group of patients at a high risk for disease relapse.

PROGNOSIS AND PREDICTION

Prognostic factors are characteristics that provide information about the likely outcome of a disease. Such prognostic markers are helpful for identifying patients with cancer who are at high risk for developing distant metastases and are therefore potential candidates for adjuvant chemotherapy. Predictive factors are characteristics that provide information about the likely benefit from treatment. Such predictive factors can be used to identify subpopulations of patients who are most likely to benefit from a given therapy. In stage II and III colon cancer, both prognostic and predictive factors are needed to refine the selection of patients for adjuvant chemotherapy.

CLINICALLY USED PROGNOSTIC AND PREDICTIVE MARKERS

TNM classification

The TNM (tumor, node, metastases) staging system developed by the AJCC (American Joint Committee on Cancer) and the IUCC (International Union for Cancer Control) remains the most important indicator for prognosis, long-term survival stratification, and

treatment guidelines in CRC[8]. Unfortunately, the system has several limitations, such as a reliance on surgical resection, which is not applicable to inoperable candidates; its inability to incorporate data on resection margins as well as molecular data (MSI and KRAS-status); and its inability to predict heterogeneous outcomes and responses to therapy with same-stage tumors.

In addition, other histopathologic features have been correlated with prognosis. These including the following:

- Lymphovascular invasion: associated with a higher risk of local recurrence, nodal disease, development of liver metastases[9-11]
- Tumor grade: higher grade associated with increased nodal involvement, metastases, recurrence after excision, and worse long-term prognosis[10,12-14]
- Histologic subtype: medullary type associated with reduced nodal involvement and better survival[15]; signet ring and small cell morphology associated with poor prognosis[16,17], as is undifferentiated type [18].

Microsatellite instability

Microsatellite instability (MSI), a form of genetic instability underlying about 15% of sporadic CRCs, is most commonly caused by loss of function of the DNA mismatch repair system (MMR)[19]. There are five human MMR genes – MLH1, MSH2, MSH6, PMS1, and PMS2 – that can be inactivated due to deletions, point mutations, or epigenetic silencing. MSH2 and MLH1 are the most common MMRs associated with Lynch syndrome, accounting for 2–3% of all CRC cases[20-22]. MSI tumors can be divided into high (MSI-H) and low/stable (MSI-L/S) subtypes based on the degree of instability observed.

MSI, regardless of subtype, is associated with better survival and lower recurrence risk after colon resection[23] and has also been correlated with chemotherapy response. MSI tumors with a N0/N1 lymph node status and proximal location are associated with improved survival with adjuvant FOLFOX chemotherapy[24]. They also show a higher response rate to bevacizumab chemotherapy whereas MSI stable disease shows no survival benefit[25]. MSI-H tumors exhibit a unique clinicopathologic pattern including proximal location, poor differentiation, histologic heterogeneity, increased lymphocytic response and inflammatory reaction, mucin production, and exophytic growth pattern[26-28]. MSI-H lesions are also associated with a lower risk of metastases and improved prognosis[29,30]

KRAS

Half of colorectal tumors are initiated by mutations in the *KRAS* oncogene. The ras family of proteins (H-, K-, and N-Ras) is associated with functions including cell growth, proliferation, and differentiation[31,32]. *KRAS*-positive mutation status is associated with decreased survival in patients undergoing bevacizumab chemotherapy[31]. The same ef-

fect has been seen in patients with a positive *NRAS* mutation (present in approximately 3% of CRCs)[33]. The National Comprehensive Cancer Network recommends screening for both *KRAS* and *NRAS* mutations before treatment with anti-EGFR (epidermal growth factor receptor) therapy[34].

Gene expression profiles

Gene expression profiling (GEP) is an emerging tool. This methodology attempts to identify differentially expressed subsets of genes (gene signatures) in groups of patients with distinct clinical outcomes.

The *Oncotype DX Colon Cancer Test* is a commercially available gene expression profile derived from real-time polymerase chain reaction. Gene expression data from 1851 patients were used to build the assay. The final panel consists of seven genes associated with recurrence for a recurrence score and six genes predictive of fluorouracil/folonic acid treatment benefit to provide a treatment score[35,36]. In several validation studies, the prognostic aspect of the test was confirmed. The predictive GEP, however, failed to significantly predict treatment response[37-39].

Another GEP assay is the *ColoPrint*, an 18-gene signature developed by Agendia. It was developed using a set of 188 fresh-frozen tumors from stage I through IV colon cancer. The selected gene set is associated with prognosis. In a study by Maak et al., the *Coloprint* was evaluated in a group of stage II CRC patients. On multivariate analysis, *Coloprint* was the only significant parameter to predict development of metastatic disease (high-risk hazard ratio=4.28, confidence interval 1.36–13.50, p= 0.013)[40,41]. Nevertheless, these gene profiles do not give sufficient information for treatment strategies in individual patients.

FOLLOW-UP

The main cause of death from CRC is metastatic disease. As mentioned, in spite of optimal surgical treatment, between 30 and 50% of patients with stage II and III CRC will develop metastatic disease[2-4]. Unfortunately, we lack the instruments to identify these patients, and all CRC patients therefore are followed for at least 5 years after colon surgery to detect recurrence. During follow-up, blood level carcinoembryogenic antigen (CEA) measurements and radiological imaging of the liver (CT imaging or ultrasound) are performed[42,43]. It is still not clear which follow-up schedule and components will provide the best surveillance strategy[44,45]. The follow-up visits of patients with CRC are labor intensive and expensive because of the administrative and logistic work as well as the numerous diagnostic tests.

COLORECTAL LIVER METASTASES

The first presentation of colorectal metastases is most often in the liver.

Liver resection is considered to be the optimal treatment for colorectal liver metastases (CLM) with 5-year survival rates up to 67% in highly selected patients[46]. At presentation, only a minor portion of patients are eligible for resection because of widespread metastatic disease or predicted insufficient liver remnant after metastasectomy. Therefore, early detection of CLM is necessary. As a result of improvements in surgical technique, the introduction of more effective systemic chemotherapy[47-49], and the use of portal vein embolization[50,51], radiofrequency ablation[52,53], and stereotactic body radiation[54], the indications for liver resection have expanded over the past decade. Despite the good outcomes for many patients undergoing this procedure, a substantial portion will still experience early recurrence with one-year recurrence rates of CLM after liver resection up to 50%[55].

The identification of patients at high risk of disease recurrence after surgery for resectable CLM might lead to better selection of patients for this procedure. These patients should either be spared an often high-risk operation or should be treated by additional and more intensified therapy to minimize the risk of early recurrence.

OUTLINE OF THE THESIS

MATCH study

To achieve identification of CRC subtypes, related prognostic markers, and treatment outcome, a multicenter cohort study including patients with CRC undergoing curative surgery was initiated in 2007 (MATCH study). The project was a collaboration among Erasmus MC University Medical Center and seven teaching hospitals, including Franciscus Gasthuis, Elisabeth Hospital, IJsselland Hospital, Ikazia Hospital, Maasstad Hospital, Reinier de Graaf Gasthuis, and Tweesteden Hospital. Tissue samples, including normal colon tissue and tumor tissue, and clinical data were stored using standard operating procedures and case record forms.

One of the vital items in the data collection is the pathology report. As discussed earlier, the TNM classification is the most important factor in determining the therapeutic approach. In stage II CRC, adjuvant chemotherapy is not recommended[56,57]. However, the American Society of Clinical Oncology (ASCO) guidelines suggest a subdivision of stage II patients into low- and high-risk groups. In high-risk patients, adjuvant chemotherapy can be considered, but it has no place in low-risk patients[56,58]. This subdivision is based on five factors: T-stage (T4), tumor differentiation grade (poor), lymphovascular invasion (present), tumor perforation (present), and lymph node metastasis status (Nx;

less than 10 lymph nodes in the resection specimen)[56]. Because of their importance and clinical implications, the pathology reports of colon cancer specimens should include a statement regarding these five factors. The Netherlands Society of Pathology introduced the guideline "Protocol Colonrectum" in 2008[59], summarizing which factors should be included in a pathology report. Until 2012, the reporting of the five risk factors was voluntary; later on, it became mandatory[59].

In **chapter 2**, we review the pathology reports of stage II patients from the MATCH cohort to determine the accuracy and completeness regarding the five high-risk factors and their impact on overall survival.

One of the key elements of the MATCH study is the collection of fresh-frozen tissue samples with the intention of using them for molecular biomarker research. Today's state-of-the-art techniques require high-quality tissue samples[60-62], and 10% of fresh-frozen samples are unsuitable for molecular analyses. In our multicenter study, tissue samples were obtained, processed, and stored following standard operating procedures in both the university hospital (a center with experience in fresh-frozen tissue sampling with dedicated personnel) and the non-university teaching hospitals (not used to or equipped/staffed for routine fresh-frozen tissue sampling). To evaluate the quality of the fresh-frozen tissue samples from the different hospitals, we performed a random check. The RNA Integrity Number (RIN), a common standard used to assess tissue quality, was measured[63,64]. In **chapter 3**, we discuss the results of this quality control procedure.

Colorectal liver metastases

With annual 1.36 million new CRC cases worldwide, the frequent follow-up visits in an outpatient clinic are time consuming and expensive. Nevertheless, after tumor resection, follow-up is of great importance to detect liver metastases at an early stage. With early diagnosis, the prognosis of patients can be improved.

In a quest for new tools to identify early disease recurrence, we sought a marker with high sensitivity, low cost, and application outside the hospital.

A promising source for biomarker research is the urine proteome, which provides detailed information for monitoring changes in human physiology[65,66]. Urine collection is non-invasive and always available in large amounts, and the natural occurring peptide (NOP) in urine has the advantage of being easily accessible without labor-intensive sample preparation. To prove this principle in patients with CLM, we conducted a pilot study. Urine from patients with CLM and from healthy persons and patients with benign liver lesions (hepatocellular adenoma) and malignant liver lesions (hepatocellular carcinoma) was collected and analyzed by mass spectrometry. We identified peptides that could differentiate among the different liver tumors and healthy persons (unpublished work, poster presentation ESMO 2010). In **chapter 4**, we describe the in-depth identification and validation of these NOPs. The practical aspects, such as sample preparation

and sample measurements by mass spectrometry, are explained, and the building of a prognostic peptide model is discussed that enables discrimination between patients with CLM and healthy persons.

In **chapter 5**, we investigate the possibility of a combined test with our urine peptide AGP and blood level CEA to increase sensitivity for detection of CLM. Alongside better sensitivity, the test would be less expensive than liver imaging and could be performed outside the hospital, by a general practitioner, for instance. For this study, we compared the AGP urine levels and CEA blood levels between patients with CLM and healthy persons.

Over the years, the number of patients eligible for liver surgery has increased because of better imaging modalities, the introduction of effective chemotherapy, and the availability of more surgical techniques, including extensive resections with or without portal vein embolization and additional treatment with radiofrequency ablation and stereotactic body radiation[48-54]. Thus, the indications for liver resection have expanded, and the only remaining limitations are unresectable extrahepatic disease and insufficient liver remnant.

As a result of these developments, the surgical approach has shifted towards an increase in nonanatomical resections. By this approach, the amount of residual liver parenchyma can be optimized, thus reducing the risk for progressive liver failure and postoperative complications[67,68]. When intrahepatic recurrence occurs, repetitive local treatment may be offered[69].

Although it has been reported that anatomical resection improves (disease-free) survival in patients with hepatocellular carcinoma[70-72], the literature about CLM is inconsistent. In **chapter 6**, we use a clinical database to investigate whether a difference can be found in surgical and oncological morbidity and overall survival comparing anatomical resections with nonanatomical resections for CLM.

A significant number of patients will not benefit from liver metastasis surgery because early recurrence will occur. As noted, the one-year recurrence rate after liver surgery for CLM can be as high as 50%[55]. Identification of patients at high risk for disease recurrence after liver resection is important because these patients could be spared an operation and might be offered chemotherapy to prevent extension of disseminated disease. To focus on this topic, we studied the feasibility of detecting circulating cells as a biomarker for recurrence.

Circulating tumor cells (CTCs) shed into the vasculature from a tumor and can be detected in the majority of patients with metastatic cancer[73]. One of the techniques for isolating these cells is the CellSearch Technique (Veridex LLC, Raritan NJ, USA). Using this method, the presence of CTCs in peripheral blood of patients has strong prognostic value in various malignancies including CRC[74-78]. Most research has been done in patients with

irresectable metastatic disease and treated with chemotherapy. Little is known about the prognostic value of CTC in patients with isolated CLM.

In **chapter 7**, we discuss the results of a pilot study to evaluate the best protocol for blood sample collection and preparation using the CellSearch System. The sampling procedure must be optimized to allow detection of small numbers of CTCs in limited disease. Subsequently, in **chapter 8**, we investigate whether the detection of CTCs by the CellSearch System can identify the high-risk patients who will develop disease recurrence within one year after liver surgery.

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Chapter 2

Completeness of Pathology Reports in Stage II Colorectal Cancer

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ABSTRACT

Introduction: The completeness of the pathological examination of resected colon cancer specimens is important for further clinical management. We reviewed the pathological reports of 356 patients regarding the five factors (pT-stage, tumor differentiation grade, lymphovascular invasion, tumor perforation and, lymph node metastasis status) that are used to identify high-risk stage II colon cancers, as well as their impact on overall survival (OS).

Methods: All patients with stage II colon cancer who were included in the first five years of the MATCH study (July 1 2007 –July 1 2012) were selected (*n*=356). The hazard ratios of relevant risk factors were calculated using Cox Proportional Hazards analyses.

Results: In as many as 69.1% of the pathology reports, the desired information on one or more risk factors was considered incomplete. In multivariable analysis, age (HR: 1.07, 95%CI 1.04-1.10, $p<0.001$), moderately- (HR: 0.35, 95%CI 0.18-0.70, $p=0.003$) and well (HR 0.11, 95% CI 0.01-0.89, $p=0.038$) differentiated tumors were significantly associated with OS.

Conclusion: Pathology reports should better describe the five high-risk factors, in order to enable proper patient selection for further treatment. Chemotherapy may be offered to stage II patients only in select instances, yet a definitive indication is still unavailable.

INTRODUCTION

Colorectal cancer is currently the second most common malignancy in the Western world[1]. Overall, 50-60% of the patients diagnosed with colorectal cancer will develop metastases[2-6]. The risk of developing metastases as well as survival can be estimated more accurately for the individual patient by taking into consideration the American Joint Committee on Cancer (AJCC) TNM classification[7]. The pathological TNM classification is the most important factor to determine the therapeutic approach[8].

For colon cancer, curatively resectable tumors are divided into AJCC stage I to III, with stage III necessitating adjuvant chemotherapy in addition to watchful waiting strategies. Patients with stage II colon carcinoma are thought not to require adjuvant chemotherapy in most cases[9,10]. However, the American Society of Clinical Oncology (ASCO) guidelines propose a subdivision of stage II patients into low and high-risk. This subdivision is based on five high-risk factors: T-stage, tumor differentiation grade, lymphovascular invasion (LVI), tumor perforation and, most importantly, lymph node metastasis status[9]. In high-risk patients, adjuvant chemotherapy can be considered, while adjuvant chemotherapy has no place in the treatment of low risk stage II colon cancer patients[9,11].

Because of their importance and clinical implications, the pathology reports of colon cancer specimens should include a statement regarding the five aforementioned factors. In addition to the five high-risk factors, molecular subtypes of cancer have been previously reported to have an effect on overall survival (OS) as well[12]. In particular, patients with microsatellite instability (MSI) are reported to have higher OS[12]. To optimize the accuracy of pathology reports on colorectal cancer specimens, the Dutch federation for pathology in 2008 drew up the guideline 'Protocol Colonrectum', summarizing which factors should be included in a pathology report and how[13]. The reporting of the five high-risk factors was facultative until early 2012, when the reporting of the factor LVI became mandatory[13].

In this study a cohort of 356 patients was reviewed to determine the accuracy and completeness regarding the five factors used to identify high-risk stage II colon cancers. We performed a detailed analysis of nodal status, which is considered the most important risk factor[6].

METHODS

Patient selection

Patients were selected from the MATCH study (MEC-2007-088), an ongoing prospective registration cohort including all patients who undergo curative surgery for primary colorectal cancer in seven hospitals in the Rotterdam region. All patients with stage II

colon cancer who were included in the first five years of the MATCH study (July 1 2007 –July 1 2012) were selected. All patients gave written informed consent.

Scoring pathology report

Pathology reports were examined for the existence of a statement on the five factors used to identify high-risk stage II patients: pathological T-stage (pT-stage), N-stage, tumor differentiation grade, LVI and tumor perforation. For the T-stage, tumor differentiation grade and LVI the presence or absence of a statement regarding these tumor characteristics was scored. For the N-stage, patients with more than 10 harvested lymph nodes were considered to have an N0 stage, while patients with less than 10 harvested lymph nodes were considered to have an Nx stage. In Nx patients, the presence or absence of a specific comment regarding the low total lymph node yield was scored. Tumor perforation was planned only to be scored present or absent in case of a clinical suspicion for perforation. As no patients had a clinical suspicion of tumor perforation, this factor was not scored. Data on MSI were not routinely scored. However, since MSI is highly correlated with right-sidedness of the tumor, we used this as a dummy variable[12]. Pathological risk factors associated with lower survival in stage II patients were individually examined in our patient cohort for both differences in clinicopathologic characteristics of patients, as well as survival analyses.

Statistical analysis

Summary statistics were provided as percentages of categorical variables and medians with interquartile ranges of continuous variables. Comparison of categorical variables was performed using the Pearson χ^2 test, while continuous variables were compared using the Kruskal-Wallis test. OS estimates and figures were created using the Kaplan-Meier method. Patients who expired within 3 months postoperatively were excluded from the survival analysis. Differences in survival amongst the different risk factors were assessed using the Log-Rank test. The hazard ratios of relevant risk factors, along with their 95% CIs, were calculated using Cox Proportional Hazards analyses. Conditional backwards selection with all relevant risk factors was conducted, based on the probability of the likelihood-ratio statistic based on conditional parameter estimates. All analyses were carried out with SPSS 22 (IBM, New York). All tests were 2-sided and $p < .05$ was considered statistically significant.

RESULTS

Pathology reports

As shown in Figure 1, in 69.1% of the pathology reports the information on one or more risk factors was considered incomplete (61.2% 1 factor, 7.9% 2 factors). T-stage and N-stage were reported in all cases. However, in the 44 Nx patients, the pathology report did not comment on this total yield as being a risk factor. In 62.8% of all cases no statement regarding presence or absence of LVI was recorded; tumor differentiation grade was not reported in 2%. As mentioned in the introduction, the reporting of LVI became mandatory in 2012. LVI, regardless whether present or absent, was reported significantly more after mandating the reporting of this risk factor (33.5% *vs*. 87.5%; $p<0.001$).

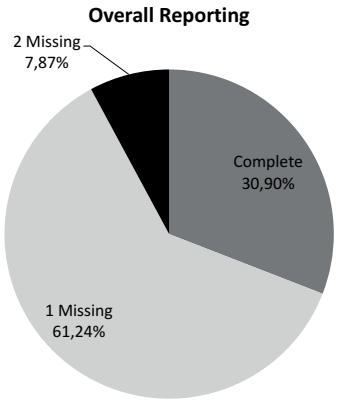


Figure 1A Overall reporting of high-risk factors (N-stage, T-Stage, Lymphovascular Invasion, and Tumor Differentiation) in the pathology report

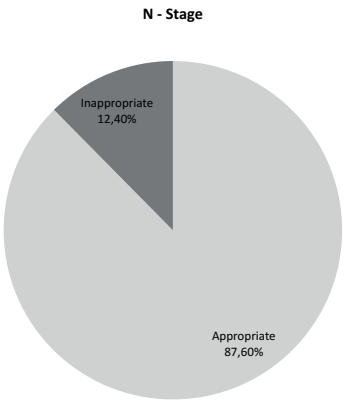


Figure 1B N-Stage Pathology Report Scoring

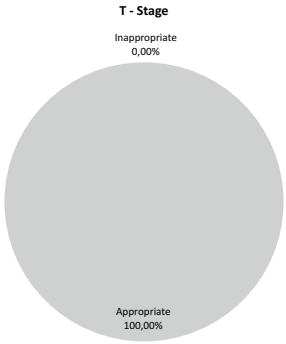


Figure 1C T-Stage Pathology Report Scoring

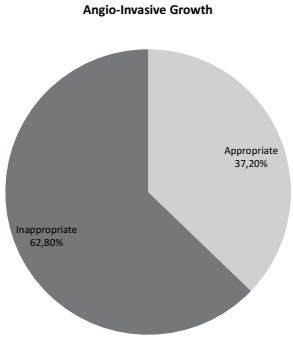


Figure 1D Lymphovascular Invasion Pathology Report Scoring

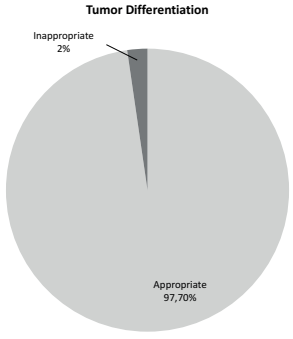


Figure 1E Tumor Differentiation Pathology Report Scoring

Clinicopathological Characteristics

Total baseline and other characteristics compared by N-stage are shown in table 1. Just over half (n=193, 54.2%) of the patients were male. The median age was 71 years (IQR 64-79 years). A diagnostic colonoscopy was performed in 327 (96.2%) of the patients and 320 (89.9%) patients underwent staging CT imaging. A small subgroup of patients received additional abdominal ultrasound (*n*=104, 29.2%), MRI (*n*=8, 2.2%), or PET-scan (*n*=1, 0.3%). More than two thirds of patients had an American Society of Anesthesiologists (ASA) classification score of 2 (*n*=195, 70.1%). At the time of surgery half of the patients underwent

Table 1: Clinicopathological Characteristics, Stratified by Nodal Status

Characteristic	N0 (n = 312)	Nx (n = 44)	P-value	Total (n = 356)
Gender				
Female	139 (44.6)	24 (54.5)		163 (45.8)
Male	173 (55.4)	20 (45.5)	0.213	193 (54.2)
Age, years (IQR)	71 (63-78)	75 (66-82)	0.029	71 (64-79)
Diabetes	51 (16.9)	2 (4.7)	0.037	53 (15.4)
Colonoscopy	285 (96.3)	42 (95.5)	0.789	327 (96.2)
Abdominal Ultrasound	94 (30.1)	10 (22.7)	0.312	104 (29.2)
CT-Abdomen	279 (89.4)	41 (93.2)	0.439	320 (89.9)
MRI Abdomen	7 (2.2)	1 (2.3)	0.990	8 (2.2)
PET-scan	1 (0.3)	0 (0.0)	0.707	1 (0.3)
ASA Class				
1	31 (12.9)	1 (2.7)		32 (11.5)
2	167 (69.3)	28 (75.7)		195 (70.1)
3	43 (17.8)	8 (21.6)		51 (18.3)
4	0 (0.0)	0 (0.0)	0.191	0 (0.0)
Type of Operation				
Open Resection	153 (49.5)	25 (58.1)		178 (50.6)
Laparoscopic Resection	156 (50.5)	18 (41.9)	0.289	174 (49.4)
Type of Resection				
Left-Sided Resection	144 (46.8)	25 (58.1)		169 (48.1)
Right-Sided Resection	154 (50.0)	18 (41.9)		172 (49.0)
(Sub)total Colectomy	10 (3.2)	0 (0.0)	0.232	10 (2.8)
AJCC T-Stage				
T3	283 (90.7)	41 (93.2)		324 (91.0)
T4	29 (9.3)	3 (6.8)	0.591	32 (9.0)
Tumor Differentation				
Poor	32 (10.3)	4 (9.1)		36 (10.1)
Moderate	260 (83.6)	38 (86.4)		298 (83.9)
Well	12 (3.9)	1 (2.3)		13 (3.7)
Unknown	7 (2.3)	1 (2.3)	0.949	8 (2.3)
Lymphovascular Invasion				
No	91 (29.3)	12 (27.3)		103 (29.0)
Yes	23 (7.4)	6 (13.6)		29 (8.2)
Unknown	197 (63.3)	26 (59.1)	0.368	223 (62.8)
Adjuvant Therapy	18 (5.8)	3 (6.8)	0.782	21 (5.9)

laparoscopic surgery (*n*=174, 49.0%) and most patients underwent a right (*n*=172, 49.0%) or left sided hemicolectomy (*n*=169, 48.1%). The majority of patients had a T3 (*n*=324, 91.0%), whereas a small minority had a T4 tumor (*n*=32, 9.0%). Of our 356 patients, 312 (87.6 %) patients did not have lymph node metastases and had more than 10 nodes, while 44 (12.4%) did not have the required minimum of 10 nodes. Over three quarters of the patients had a moderately differentiated tumor (*n*=298, 83.9%). A small subgroup of patients (*n*=21, 5.9%) received adjuvant therapy. 13 patients (4.1%) expired within 90 days and were therefore excluded in survival analyses.

Differences between the patients with and without high-risk factors were more closely evaluated (table 1, supplementary table 1-3). Between the Nx and N0 group a significant difference was observed in median age, with the Nx group being significantly older (71 *vs*. 75 years, p=0.037). T4 patients as opposed to T3 patients received adjuvant chemotherapy (37.5% *vs*. 2.8%, p<0.001) more often, and had an unknown (not reported) differentiation grade of their tumor relatively more frequently (9.4% *vs*. 1.5%, p=0.021). No clinical differences were observed between patients with demonstrated LVI, patients without LVI, and patients in whom this factor was not recorded in the pathology report. Finally, there was a trend towards administering chemotherapy in patients with worse tumor differentiation (p=0.086).

Overall Survival per High-risk Factor

Median follow-up in our cohort was 72.4 months (IQR 62.8-80.8). The 1-, 3- and 5-year survival was 98.0 %, 89.1, and 80.4% respectively. When examining high-risk factors more closely in our cohort of stage II patients we found that these risk factors did not seem to have a significant impact on survival (figure 2). In our study, we did not find a significant association between the failure to report any of the five factors and overall survival. No difference was found between left-sided colorectal cancer, right-sided colorectal cancer, and colorectal cancer on both sides. As depicted in the Kaplan Meier graphs, no trend towards a difference was visible for any of the risk factors either. Age (HR: 1.06, 95% CI 1.03-1.08, p<0.001) and ASA Class 3 (HR: 3.52, 95% CI 1.19-10.42, p=0.023) were significantly associated with OS in univariable analysis. In multivariable analysis age (HR: 1.07, 95% CI 1.04-1.10, p<0.001), moderately- (HR: 0.35, 95% CI 0.18-0.70, p=0.003) and well (HR 0.11, 95% CI 0.01-0.89, p=0.038) differentiated tumors were significantly associated with OS, after conditional backwards selection of associated variables (Table 2).

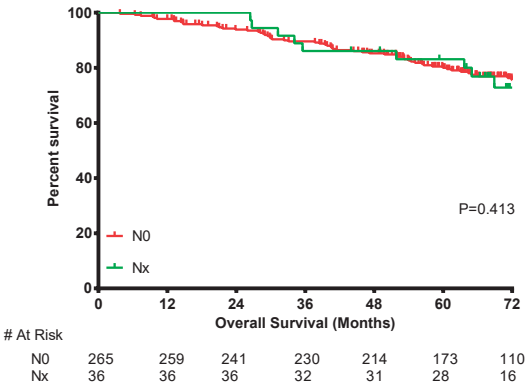
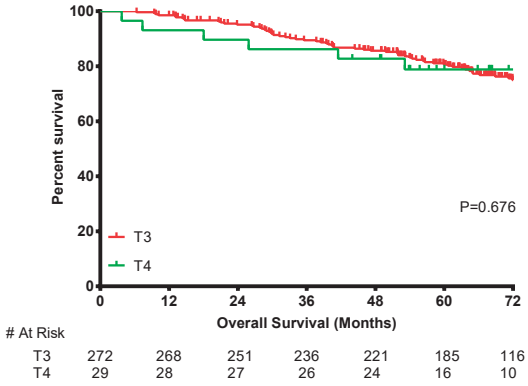


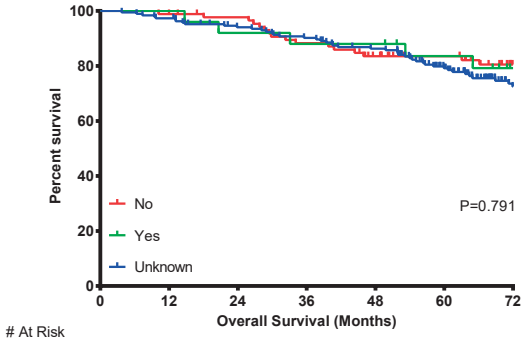
Figure 2A Overall survival stratified by N-Stage



At Risk

T3	272	268	251	236	221	185	116
T4	29	28	27	26	24	16	10

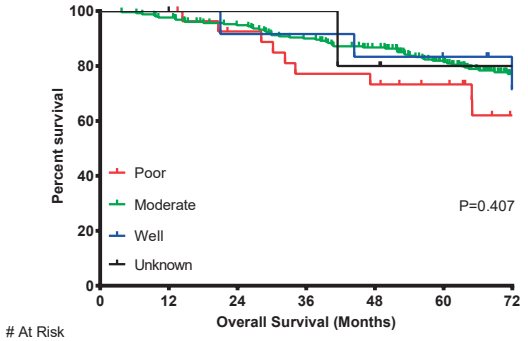
Figure 2B Overall survival stratified by T-Stage



At Risk

No	88	87	84	76	68	60	42
Yes	25	25	24	23	23	20	15
Unknown	187	183	171	164	155	122	70

Figure 2C Overall survival stratified by lymphovascular Invasion



At Risk

Poor	28	28	25	21	20	17	10
Moderate	254	249	236	224	210	173	108
Well	12	12	11	11	10	10	7
Unknown	6	6	6	6	5	3	3

Figure 2D Overall survival stratified by Differentiation Grade

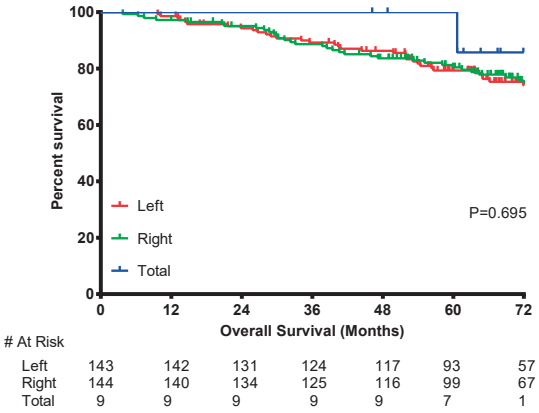


Figure 2E Overall survival stratified by Type of Colectomy

Table 2. Survival Analysis of Stage II Colon Cancer Patients

	Univariable Analysis			Multivariable Analysis		
Characteristic	**HR**	**95% CI**	**p value**	**HR**	**95% CI**	**p value**
Age, years (IQR)	1.06	1.03-1.08	<0.001	1.07	1.04-1.10	<0.001
AJCC T-Stage						
T3	Ref	-	-			
T4	0.84	0.36-1.93	0.677			
N-Stage						
N0	Ref	-	-			
Nx	1.29	0.70-2.39	0.414			
Tumor Differentiation						
Poor	Ref	-	-	Ref	-	-
Moderate	0.57	0.29-1.11	0.097	0.35	0.18-0.70	0.003
Well	0.58	0.16-2.11	0.409	0.11	0.01-0.89	0.038
Lymphovascular Invasion						
No	Ref	-	-			
Yes	1.31	0.58-2.97	0.522			
Unknown	1.14	0.68-1.93	0.617			
Diabetes						
No	Ref	-	-			
Yes	1.25	0.71-2.20	0.446			
Colonoscopy						
No	Ref	-	-			
Yes	1.33	0.32-5.42	0.694			
ASA Class						
1	Ref	-	-			
2	1.60	0.57-4.46	0.371			
3	3.52	1.19-10.42	0.023			

Table 2. Survival Analysis of Stage II Colon Cancer Patients (*continued*)

	Univariable Analysis			Multivariable Analysis		
Characteristic	**HR**	**95% CI**	**p value**	**HR**	**95% CI**	**p value**
Type of Operation						
Open Resection	Ref	-	-			
Laparoscopic Resection	1.13	0.72-1.77	0.605			
Type of Resection						
Left-Sided Resection	Ref	-	-			
Right-Sided Resection	1.03	0.65-1.61	0.913			
(Sub)total Colectomy	0.44	0.06-3.23	0.422			
Adjuvant Therapy	0.42	0.10-1.71	0.224			

DISCUSSION

Colorectal cancer is currently one of the most common malignancies in the Western world[1]. For colon cancer, tumors are divided into stage I to IV with a subdivision for stage II patients into low and high-risk patients, based on the factors pT-stage, tumor differentiation grade, LVI, tumor perforation and, most importantly, lymph node metastasis status. In this study a set of 356 pathology reports was reviewed to determine the accuracy and completeness regarding the five factors used to identify high-risk stage II colon cancers and the impact on clinical management.

In 2007 Quirke *et al.* suggested three main reasons for the incompleteness of pathology: the ignorance of the importance of certain features for clinical management, the large number of possible prognostic features that could be reported, and the desire to hold on to free text reports[14]. While the first may be overcome by education and routine audit with feedback, the second and third reason requires a standardized minimum set of items that should be reported. Interestingly, in 1998 the Royal College of Pathologists already suggested such a set which included all five factors examined in the current study[15]. The use of proforma reporting for pathology reports on colorectal cancer specimens has been described to increase the completeness of the reports up to 96%[16,17]. Synoptic reporting, in which a prespecified set of items have to be scored before the report can be finalized, has also been described to add to the completeness of pathology reports[18]. The increase of LVI reporting in our data after synoptic reporting became mandatory substantiates these earlier observations.

In 2000, the college of American Pathologists published a statement summarizing and categorizing the pathologic prognostic factors and predictive factors in colorectal cancer.[19] The pT category and pN category of the pTNM staging system as well as LVI were categorized in Category I, which included factors definitively proven to be of prognostic import based on evidence from multiple statistically and methodologically

well executed published trials. Tumor grade fell into Category IIA, which included factors extensively studied clinically and/or biologically and repeatedly shown to have prognostic and/or predictive value, but has to be validated in statistically robust studies. The importance to mention these tumor characteristics was illustrated by Maughan *et al.*, who reported an association between the failure to report either vascular invasion or peritoneal involvement and overall survival in a large retrospective study of close to 6,000 patients[20].

In our cohort, reporting of most high-risk factors in stage II patients was absent in the majority of the pathology reports between 2007 and 2012. However, a difference in OS between patients in whom factors indicating worse prognosis were not reported and those in whom they were absent was not found. Poorly differentiated tumors, performed worse than moderately and well differentiated tumors when corrected for age at the time of surgery. Age itself was an independent risk factor as well. The reasons for the lack of predictive value of the other four recognized high-risk factors in our cohort is likely multifactorial. Firstly, this was a prospectively included cohort in which all variables were scored before the individual disease course of patients was known, eliminating potential bias in the scoring of variables. All patients were demographically similar, as they were treated in the same region in the Netherlands. This also limited differences in the quality of health care potentially correlating with the quality of diagnosis.

The use of adjuvant chemotherapy in stage II colon cancer patients remains open for discussion. Current literature does not support the use of adjuvant chemotherapy for all stage II colon cancer patients since it does not improve disease-free or overall survival as illustrated in the MOSAIC trial[21]. However, the indirect evidence of the beneficial role of adjuvant chemotherapy in stage III colon cancer patients and the identification of high-risk stage II colon cancer patients using the currently available risk factors justifies the consideration of the adjuvant chemotherapy as stated by the American Society of Clinical Oncology[9]. In our study the use of adjuvant chemotherapy was not a significant predictor of better overall survival (HR: 0.42, 95% CI: 0.10-1.71, $p = 0.224$).

Although it is, to our knowledge, the first study into the completeness of prognostic factor reporting in type II colon cancer patients, this study has a number of limitations. First of all, our number of patients is comparatively low. Because our study is based on primary data of patients with stage II colon cancer, however, this is one of the larger studies of its kind[6,14-16]. A direct consequence of cohort size is a relatively low number of patients within some subgroups of the survival analysis. Since selection bias is highly unlikely due to the prospective inclusion of this cohort, we have no reason to question the accuracy of our data. An increase in patient numbers, therefore, would only lead to improved precision (i.e. smaller confidence intervals) and would not change the trends in survival depicted by our data. We therefore believe that an increase of the number

of patients in the subgroups would still not lead us to conclude clinically significant differences over these subgroups, with regard to overall survival.

We conclude that pathology reports should better describe the five high-risk factors in stage II colon cancer, in order to enable proper patient selection for further treatment. Of the five factors only the tumor differentiation grade was observed to be prognostic in multivariable survival analysis. Chemotherapy may be offered to patients only in select instances, when a certain set of prognostic markers is present. Further research into these prognostic markers is warranted, as a definitive set is still unavailable.

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Supplementary Table 1: Clinicopathological Characteristics, Stratified by T Stage

Characteristic	T3 (n = 324)	T4 (n = 32)	P-value	Total (n = 356)
Gender				
Female	149 (46.0)	14 (43.8)		163 (45.8)
Male	175 (54.0)	18 (56.2)	0.808	193 (54.2)
Age, years (IQR)	72 (63-79)	71 (65-76)	0.587	71 (64-79)
Diabetes	50 (16.0)	3 (9.4)	0.324	53 (15.4)
Colonoscopy	297 (96.1)	30 (96.8)	0.856	327 (96.2)
Abdominal Ultrasound	92 (28.4)	12 (37.5)	0.280	104 (29.2)
CT-Abdomen	289 (89.2)	31 (96.9)	0.169	320 (89.9)
MRI Abdomen	8 (2.5)	0 (0.0)	0.369	8 (2.2)
PET-scan	1 (0.3)	0 (0.0)	0.753	1 (0.3)
ASA Class				
1	28 (11.1)	4 (15.4)		32 (11.5)
2	175 (69.4)	20 (76.9)		195 (70.1)
3	49 (19.4)	2 (7.7)		51 (18.3)
4	0 (0.0)	0 (0.0)	0.311	0 (0.0)
Type of Operation				
Open Resection	162 (50.3)	16 (53.3)		178 (50.6)
Laparoscopic Resection	160 (49.7)	14 (46.7)	0.751	174 (49.4)
Type of Resection				
Left-Sided Resection	157 (48.9)	12 (40.0)		169 (48.1)
Right-Sided Resection	156 (48.6)	16 (53.3)		172 (49.0)
(Sub)total Colectomy	8 (2.5)	2 (6.7)	0.324	10 (2.8)
Tumor Differentiation				
Poor	34 (10.5)	2 (6.2)		36 (10.1)
Moderate	271 (83.9)	27 (84.4)		298 (83.9)
Well	13 (4.0)	0 (0.0)		13 (3.7)
Unknown	5 (1.5)	3 (9.4)	0.021	8 (2.3)
Lymphovascular Invasion				
No	96 (29.7)	7 (21.9)		103 (29.0)
Yes	27 (8.4)	2 (6.2)		29 (8.2)
Unknown	200 (61.9)	23 (71.9)	0.539	223 (62.8)
N-Stage				
N0	283 (87.3)	29 (90.6)		312 (87.6)
Nx	41 (12.7)	3 (9.4)	0.591	44 (12.4)
Adjuvant Therapy	9 (2.8)	12 (37.5)	<0.001	21 (5.9)

Supplementary Table 2: Clinicopathological Characteristics, Stratified by Lymphovascular Invasion

Characteristic	No Lymphovascular Invasion (n = 103)	Lymphovascular Invasion (n = 29)	Unknown (n = 223)	P-value	Total (n = 356)
Gender					
Female	48 (46.6)	13 (44.8)	102 (45.7)		163 (45.8)
Male	55 (53.4)	16 (55.2)	121 (54.3)	0.982	193 (54.2)
Age, years (IQR)	69 (61-76)	71 (62-79)	72 (65-80)	0.049	71 (64-79)
Diabetes	16 (16.2)	3 (10.3)	33 (15.3)	0.740	53 (15.4)
Colonoscopy	90 (91.8)	29 (100.0)	207 (97.6)	0.025	327 (96.2)
Abdominal Ultrasound	24 (23.3)	7 (24.1)	73 (32.7)	0.180	104 (29.2)
CT-Abdomen	94 (91.3)	25 (86.2)	200 (89.7)	0.721	320 (89.9)
MRI Abdomen	1 (1.0)	1 (3.4)	6 (2.7)	0.563	8 (2.2)
PET-scan	0 (0.0)	0 (0.0)	1 (0.4)	0.743	1 (0.3)
ASA Class					
1	11 (13.8)	2 (9.1)	19 (10.9)		32 (11.5)
2	57 (71.2)	16 (72.7)	121 (69.1)		195 (70.1)
3	12 (15.0)	4 (18.2)	35 (20.0)		51 (18.3)
4	0 (0.0)	0 (0.0)	0 (0.0)	0.856	0 (0.0)
Type of Operation					
Open Resection	56 (56.0)	18 (62.1)	103 (46.4)		178 (50.6)
Laparoscopic Resection	44 (44.0)	11 (37.9)	119 (53.6)	0.119	174 (49.4)
Type of Resection					
Left-Sided Resection	42 (42.0)	12 (41.4)	115 (52.0)		169 (48.1)
Right-Sided Resection	52 (52.0)	17 (58.6)	102 (42.6)		172 (49.0)
(Sub)total Colectomy	6 (6.0)	0 (0.0)	4 (1.8)	0.092	10 (2.8)
AJCC T-Stage					
T3	96 (93.2)	27 (93.1)	200 (89.7)		324 (91.0)
T4	7 (6.8)	2 (6.9)	23 (10.3)	0.539	32 (9.0)
Tumor Differentiation					
Poor	10 (9.7)	7 (24.1)	19 (8.6)		36 (10.1)
Moderate	86 (83.5)	20 (69.0)	192 (86.5)		298 (83.9)
Well	3 (2.9)	1 (3.4)	8 (3.6)		13 (3.7)
Unknown	4 (3.9)	1 (3.4)	3 (1.4)	0.153	8 (2.3)
N-Stage					
N0	91 (88.3)	23 (79.3)	197 (88.3)		312 (87.6)
Nx	12 (11.7)	6 (20.7)	26 (11.7)	0.368	44 (12.4)
Adjuvant Therapy	5 (4.9)	4 (13.8)	12 (5.4)	0.169	21 (5.9)

Supplementary Table 3: Clinicopathological Characteristics, Stratified by Tumor Differentiation

Characteristic	Poor (n = 36)	Moderate (n = 298)	Well (n = 13)	Unknown (n= 8)	P-value	Total (n = 356)
Gender						
Female	17 (47.2)	136 (45.6)	7 (53.8)	2 (25.0)		163 (45.8)
Male	19 (52.8)	162 (54.4)	6 (46.2)	6 (75.0)	0.623	193 (54.2)
Age, years (IQR)	69 (58-76)	64 (72-79)	70 (68-77)	65 (55-77)	0.257	71 (64-79)
Diabetes	3 (8.3)	44 (15.3)	6 (46.2)	0 (0.0)	0.007	53 (15.4)
Colonoscopy	35 (100.0)	270 (95.4)	13 (100.0)	8 (100.0)	0.444	327 (96.2)
Abdominal Ultrasound	12 (33.3)	85 (28.5)	5 (38.5)	2 (25.0)	0.809	104 (29.2)
CT-Abdomen	29 (80.6)	272 (91.3)	10 (76.9)	8 (100.0)	0.061	320 (89.9)
MRI Abdomen	2 (5.6)	5 (1.7)	1 (7.7)	0 (0.0)	0.245	8 (2.2)
PET-scan	0 (0.0)	1 (0.3)	0 (0.0)	0 (0.0)	0.979	1 (0.3)
ASA Class						
1	6 (18.8)	23 (10.2)	1 (8.3)	2 (28.6)		32 (11.5)
2	17 (53.1)	164 (72.6)	9 (75.0)	4 (57.1)		195 (70.1)
3	9 (28.1)	39 (17.3)	2 (16.7)	1 (14.3)		51 (18.3)
4	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0.296	0 (0.0)
Type of Operation						
Open Resection	22 (61.1)	144 (49.0)	6 (46.2)	6 (75.0)		178 (50.6)
Laparoscopic Resection	14 (38.9)	150 (51.0)	7 (53.8)	2 (25.0)	0.272	174 (49.4)
Type of Resection						
Left-Sided Resection	12 (33.3)	150 (51.2)	4 (30.8)	2 (25.0)		169 (48.1)
Right-Sided Resection	24 (66.7)	135 (46.1)	8 (61.5)	5 (62.5)		172 (49.0)
(Sub)total Colectomy	0 (0.0)	8 (2.7)	1 (7.70	1 (12.5)	0.059	10 (2.8)
AJCC T-Stage						
T3	34 (94.4)	271 (90.9)	13 (100.0)	5 (62.5)		324 (91.0)
T4	2 (5.6)	27 (9.1)	0 (0.0)	3 (37.5)	0.021	32 (9.0)
Lymphovascular Invasion						
No	10 (27.8)	86 (28.9)	3 (25.0)	4 (50.0)		103 (29.0)
Yes	7 (19.4)	20 (6.7)	1 (8.3)	1 (12.5)		29 (8.2)
Unknown	19 (52.8)	192 (64.4)	8 (66.7)	3 (37.5)	0.153	223 (62.8)
N-Stage						
N0	32 (88.9)	260 (87.2)	12 (92.3)	7 (87.5)		312 (87.6)
Nx	4 (11.1)	38 (12.8)	1 (7.7)	1 (12.5)	0.949	44 (12.4)
Adjuvant Therapy	3 (8.3)	16 (5.4)	0 (0.0)	2 (25.0)	0.086	21 (5.9)

Chapter 3

Multicenter fresh frozen tissue sampling in colorectal cancer; does the quality meet the standards for state of the art biomarker research?

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ABSTRACT

Introduction: The growing interest in the molecular subclassification of colorectal cancers is more and more facilitated by large multicenter biobanking initiatives.[1,2] The quality of tissue sampling is pivotal for successful translational research. This study shows the quality of fresh frozen tissue sampling within a multicenter cohort study for colorectal cancer (CRC) patients.

Material and Methods: Each of the seven participating hospitals randomly contributed ten tissue samples, which were collected following Standard Operating Procedures using established techniques. To indicate if the amount of intact RNA is sufficient for molecular discovery research and prove SOP compliance, the RNA integrity number (RIN) was determined. Samples with a RIN <6 were measured a second time and when consistently low a third time. The highest RIN was used for further analysis.

Results: 91% of the tissue samples had a RIN ≥6 (91%). The remaining six samples had a RIN between 5-6 (4.5%) or lower than 5 (4.5%). The median overall RIN was 7.3 (range, 2.9 to 9.0). The median RIN of samples in the university hospital homing the biobank was 7.7 and the median RIN for the teaching hospitals was 7.3, ranging from 6.5 to 7.8. No differences were found in the outcome of different hospitals (p=0.39).

Conclusion: This study shows that the collection of high quality fresh frozen samples of colorectal cancers is feasible in a multicenter design with complete SOP adherence. Thus, using basic sampling techniques large patient cohorts can be organized for predictive and prognostic (bio)marker research for CRC.

INTRODUCTION

Colorectal cancer (CRC) is the second most common malignancy in the Western World[3]. As in all cancer research, there is a strong trend towards molecular subclassification of CRC[4]. The studies conducted to identify these molecular and clinically relevant markers demand large numbers of patients with accurate long-term clinical data combined with high quality tissue samples to be able to use state of the art techniques[1,5,6]. Subsequently, the standard enclosed formalin-fixed paraffin-embedded tissue can be used to develop assays for daily clinical practice. Therefore, large multicenter biobanking initiatives are needed to facilitate these research efforts[1,2]. However, 10% of the fresh frozen tissue samples collected for research purposes are unsuitable for molecular analyses. This is due to multiple non-modifiable factors such as tissue type, intrinsic patient factors, warm ischemia time (extraction of the resection specimen after ligation of the large vessels) and modifiable factors such as cold ischemia time (tissue transport from the operating theatre to the pathology lab), the conservation (fixation/stabilization) method, subsequent transport and the storage of the tissue samples[7,8]. The RNA Integrity Number (RIN), first described in 2006, is currently a common standard used to assess tissue quality[9]. This method became well accepted to measure the SOP adherence of quality in tissue banking[10].

In this cohort, fresh frozen tissue samples were obtained in both a university (a center with experience in tissue sampling and storage, and dedicated personnel)[11] as well as six non-university teaching hospitals also referred to as peripheral hospitals (not used to, and equipped and staffed for routine fresh frozen tissue sampling) is assessed within the MATCH study, a multicenter cohort study in the region of Rotterdam, the Netherlands, including patients with CRC.

MATERIALS AND METHODS

MATCH-study design

The MATCH-study is an ongoing multicenter cohort study including adult patients with CRC undergoing curative surgery. The participating centers include one university hospital (Erasmus MC Medical Center) and six non-university teaching hospitals (Elisabeth hospital, IJsselland hospital, Ikazia hospital, Maasstad hospital, Reinier de Graaf Gasthuis, Franciscus Gasthuis). The MATCH study was approved by the Medical Ethical Board of the Erasmus MC Medical Center, Rotterdam, the Netherlands (MEC-2007-088). All patients provide written informed consent for the collection of long-term clinical data and storage of tissue samples. The study is of an integrated approach using clinical patient care in non-university hospitals with university-based facilities for tissue and data

storage. The rationale of this study was to identify subtypes of colorectal cancer, related prognostic markers and outcome of treatment. Liver metastases was defined as primary outcome defining a good or dismal outcome of disease progression as liver involvement has been demonstrated to be the main factor to determine long term outcome.

Clinical data

Medical specialists of departments of Surgery, Pathology, Gastroenterology, Radiology and Medical oncology were consulted. Clinical data included reports of colonoscopy, radiology and pathology, as well as, surgical reports and postoperative complications. A standard case record was created in a web based multicenter access database. The follow-up of these patients was standardized in all hospitals following an intensive follow-up schedule according the national CRC guidelines[12].

Tissue sampling

All tissue samples were handled following a Standard Operation Procedure (SOP) provided by the study team at the start of the study. In short, resection specimens were transported (at room temperature without any conservation fluids) from the operating theatre to the pathology department, immediately following removal of the specimen from the patient. At the pathology department the specimen was handled at room temperature and within two hours after resection samples were snap-frozen as described below. When the two hour time limit was exceeded, no tissue samples were taken.

Macroscopically, one to four tumor samples and one to two healthy colon tissue samples of 0.5-1 cm^3 were taken by the pathologist. Tissue sampling for the MATCH study was not allowed to interfere with the standard pathology routine needed for clinical practice. Tumor and normal tissue were stored in labeled cryovials and snap frozen in liquid nitrogen or dry-ice[11,13]. Samples were then stored at low-temperature refrigerators (-80°C) in the hospital of primary surgery and in batches transported to the central tissue bank (-196°C liquid nitrogen Barrels) at the university hospital. Of all new tissue specimens stored in the central bank, on a yearly base 2% is tested for quality, by determining the RNA integrity[10,14].

Tissue quality assessment

To assess the tissue quality of the samples collected in the MATCH-study, we randomly selected 10 tissue samples per participating hospital, representing about 4% of the entire collection. Samples that were exposed to neoadjuvant chemotherapy and/or radiotherapy were excluded as this may damage tissue resulting in failure of analysis.

RNA quality was determined by measuring of the RIN[9,15]. For RNA isolation, 10-20 tissue slides of 10µm were cut. One slide was colored by hematoxylin and eosin (H&E) stain for morphological conformation of the diagnosis. For RNA extraction, the slides were

put in a Qiazol Lysis buffer and shaken for ten seconds to homogenize the tissue. RNA was then extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the method suggested by the manufacturer. The integrity of RNA was measured by the Bioanalyser (Agilent Technologies, Santa Clara, CA, USA) using the lab-on-a-chip, RNA 6000 nano assay. This is an automated system bases on electrophoretic separation. The RIN is directly calculated applying an algorithm on the ratio of 18S/28S ribosomal RNA bands. A tissue sample with a RIN of ≥6 is believed to be of good quality (Figure 1a)[16]. Samples with a RIN <6 (Figure 1b) were measured a second and when consistently low a third time. When the RIN was consistently low, the case was discussed with the technician to see if any deviation from protocol (e.g. during the freezing procedure or sample preparation) could explain the low RIN. When samples were measured multiple times, the highest RIN was used for further analysis.

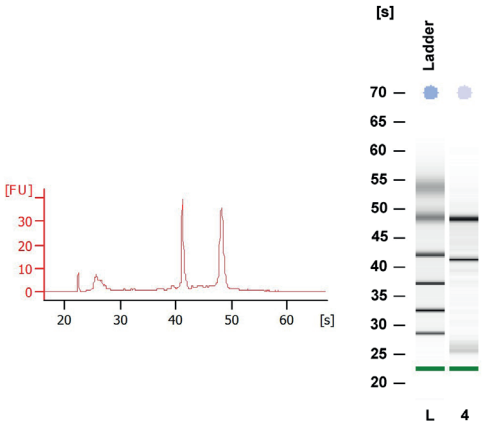

Figure 1a Image intact RNA (RIN 9.0), obtained from the electropherogram and virtual gel

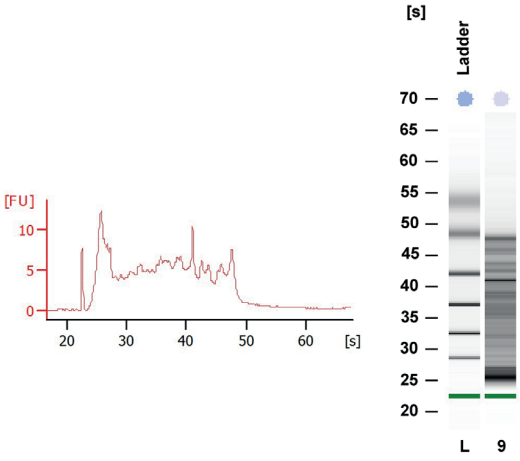

Figure 1b Image partially degraded RNA (RIN 3.3), obtained from the electropherogram and virtual gel

Statistical analysis

Statistical analyses were performed by using SPSS (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.). Categorical data were described as frequencies with percentages and continuous data as median with the range. The χ^2 test was used to compare categorical data, for continuous date the One-way ANOVA test was used. A *p*-value less than 0.05 was considered to be statistically significant.

RESULTS

In total, 70 random samples were selected for analysis out of the 1700 samples collected in the study period 1st October 2007 – 1st January 2013. During the work-up and data quality check, three samples were excluded leaving a total sample size of *n*=67. Two tissue samples were exposed to neoadjuvant radiation therapy and one tissue sample was too small.

Out of the 67 samples, two samples were analyzed two times (3.0%) and seven samples three times (10.4%). The median overall RIN of all samples was 7.3 (range, 2.9 to 9.0). The majority (*n*=61) of the tissue samples had a RIN ≥6 (91%). The remaining six samples had a RIN between 5-6 (4.5%) or lower than 5 (4.5%) (Figure 2 and 3). Three of the seven samples that were measured three times had a RIN < 5 and were discussed with the technician. However, the low RIN could not be attributed to protocol deviations. The median RIN for a center specialized in tissue sampling at the operation theatre (university hospital) was 7.7 and the median RIN for teaching hospitals without a wide experience in this field ranged from 6.5 to 7.8. The overall median RIN of the non-university teaching hospitals (median RIN =7.3) did not differ significantly with the median RIN of the

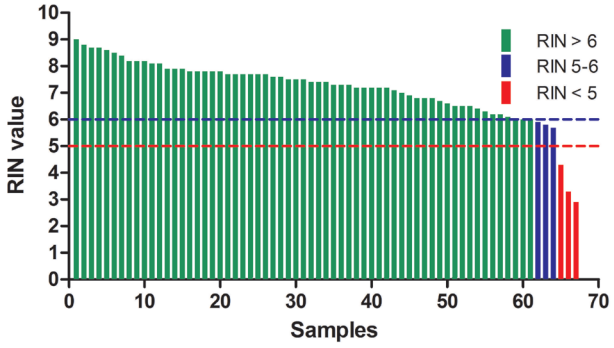

Figure 2 The RIN distribution in 67 samples

university hospital (p=0.39) (Figure 4). When using the specialized university hospital as a reference, the median RIN of one non specialized teaching hospital (hospital 6) had a significantly lower median RIN than the university hospital (p=0.02). However, a median RIN of 6.5 is still well above the cut-off of 6. Interestingly, the range of RIN for the non-university teaching hospitals tended to be larger than the range of RIN if the university hospital (Figure 3).

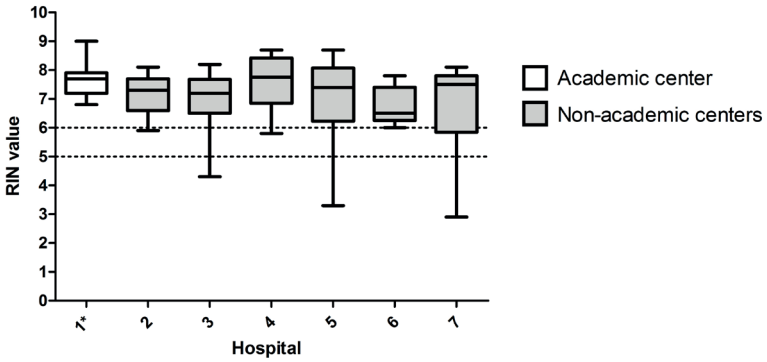

Figure 3 Box plot with the RIN per hospital

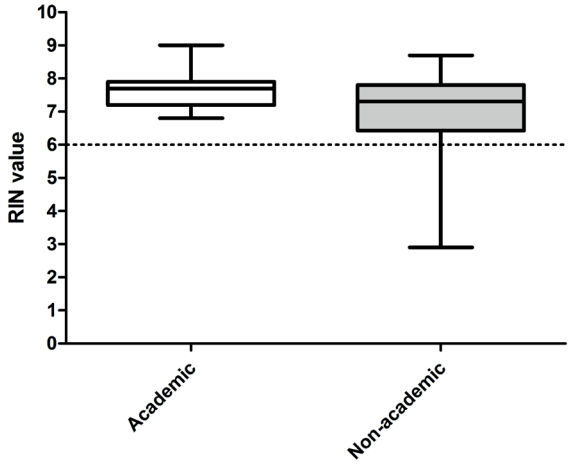

Figure 4 Box plot with the RIN for the university hospital and non-university hospitals

DISCUSSION

This study shows that the collection of high quality fresh frozen samples of CRC is feasible in a multicenter design including hospitals for which fresh frozen tissue sampling is not part of the daily routine. In our study, 91% had a RIN ≥6 and thus can be used for highly demanding gene array assays.

The RIN was developed and published in 2006 to meet the need for a reliable standard to estimate the integrity of RNA samples[9]. A comparison study comparing a subjective evaluation of the electropherogram, the 28S-18S peaks ratio and the RIN showed a superior result for the manual and RIN method over the ratio method[16]. Nowadays, the RIN is widely used to quantify the RNA quality of samples and select samples for expression analyses. However, the cut-off used to select 'high quality' samples varies in literature, ranging from a RIN of 5 to 7. These cut-offs can be based on the recommendations in a manufacturer manual or on the experience of a lab[17-20]. At our hospital, we use a RIN of ≥6 as the cut-off which qualified 91% of the samples as high quality samples[21]. When samples repeatedly have a RIN <6, they may be excluded to prevent a transcript specific bias, or analytical or bioinformatics steps specifically dealing with the low quality samples should be included in the methodology[22,23]. Furthermore, samples with a RIN < 6 can still be used for RT-qPCR applications in which only short amplicons are analyzed.

The quality of RNA expression in tissue samples is dependent on multiple factors such as tissue type, intrinsic patient factors, warm and cold ischemia time, the fixation method and the storage of the tissue samples. While tissue type and intrinsic patient factors cannot be modified, other factors (i.e. ischemia time, fixation method and the storage of samples) can be influenced. The RIN can be used to determine large influences during the pre-analytical phase. Smaller differences can be assessed based on RNA expression analyses[24]. For fresh frozen samples, the most important factor appears to be the ischemia time and freeze thawing effects after freezing. A recent review specifically addressing the effect of cold ischemia on RNA stability concluded that in most studies included only minimal changes in the RIN were observed (≤10%) during a cold ischemia times of 1-6 hours[25]. One outlier reported a significantly decreased RIN of 44% in samples with a cold ischemia time of 1,5 hours compared to samples with a cold ischemia time of 10 minutes[20]. However, the 28S:18S ratios did not significantly differ[20]. Importantly, the definition of cold ischemia time differed between studies and often the cold ischemia time in the operating theatre was not taken into account. Furthermore, the effects of warm ischemia time are often ignored while they most likely interact with the effects of cold ischemia time. This may be explained by the fact that this factor is hard to reliably score and is considered to be a non-modifiable factor since attempts to minimize warm ischemia time may affect patient care. Such non-modifiable influences can only be documented to obtain a tool for determination of this influence[26]. Although

we did not specifically assessed the association between ischemia time and the RIN in our study, the maximum cold ischemia time was two hours since this was included in the SOP. Thus, the high percentage of high quality samples in our study is in line with the current literature. For the few samples with consistently low RIN values, no protocol deviations were found suggesting the low RIN was caused by non-modifiable factors.

Our study shows that SOP compliance was positive in all the cooperating hospitals and high quality fresh frozen tissue sampling is possible in a multicenter setting including both university and non-university hospitals. These findings support the feasibility of emerging large-scale 'fit-for-purpose' biobanks to facilitate the increasingly complex field of fundamental and translational cancer research[1,2,27].

In conclusion, our study shows that the collection of high quality fresh frozen samples of CRC is feasible in a multicenter design and using basic sampling techniques. Thus, large patient cohorts can be organized for predictive and prognostic (bio)marker research for CRC.

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Chapter 4

Collagen peptides in urine: a new promising biomarker for the detection of colorectal liver metastases

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ABSTRACT

Introduction: For both patients and the outpatient clinic the frequent follow-up visits after a resection of colorectal cancer (CRC) are time consuming and due to large patient numbers expensive. Therefore, it is important to develop an effective non-invasive test for the detection of colorectal liver metastasis (CLM) which could be used outside the hospital.

The urine proteome is known to provide detailed information for monitoring changes in the physiology of humans. Urine collection is non-invasive and urine naturally occurring peptides (NOPs) have the advantage of being easily accessible without labour-intensive sample preparation. These advantages make it potentially useful for a quick and reliable application in clinical settings. In this study, we will focus on the identification and validation of urine NOPs to discriminate patients with CLM from healthy controls.

Materials and methods: Urine samples were collected from 24 patients with CLM and 25 healthy controls. In the first part of the study, samples were measured with a nano liquid chromatography (LC) system (Thermo Fisher Scientific, Germaring, Germany) coupled on-line to a hybrid linear ion trap / Orbitrap mass spectrometer (LTQ-Orbitrap-XL, Thermo Fisher Scientific, Bremen, Germany). A discovery set was used to construct the model and consecutively the validation set, being independent from the discovery set, to check the acquired model. From the peptides which were selected, multiple reaction monitoring (MRM's) were developed on a UPLC-MS/MS system.

Results: Seven peptides were selected and applied in a discriminant analysis a sensitivity of 84.6% and a specificity of 92.3% were established (Canonical correlation:0.797, Eigenvalue:1.744, F:4.49, p:0.005). The peptides AGPP(-OH)GEAGKP(-OH)GEQGVP(-OH) GDLGA P(-OH)GP and KGNSGEP(-OH)GAPGSKGDTGAKGEP(-OH)GPVG were selected for further quantitative analysis which showed a sensitivity of 88% and a specificity of 88%.

Conclusion: Urine proteomic analysis revealed two very promising peptides, both part from collagen type 1, AGPP(-OH)GEAGKP(-OH)GEQGVP(-OH)GDLGAP(-OH)GP and KGNSGEP(-OH)GAPGSKGDTGAKGEP(-OH)GPVG which could detect CLM in a non-invasive manner.

INTRODUCTION

Colorectal cancer (CRC) is the most common gastrointestinal malignancy worldwide and the 3rd leading cause of cancer-related deaths in the western world. More than one-third of the patients develop colorectal liver metastases (CLM) during the course of the disease, which are responsible for at least two-thirds of the deaths[1].

For the follow-up after CRC, blood level Carcinoembryonic antigen (CEA) is used to detect CLM with a wide spread of sensitivity ranging from 58 to 89 percent[2,3]. Because of this suboptimal sensitivity, liver imaging with ultrasonography and computer tomography are performed on a routine base. For both patients and the outpatient clinic the frequent follow-up visits are time consuming and due to large patient numbers expensive. Therefore, it is important to develop an effective non-invasive test for the detection of CLM which could be used outside the hospital.

Proteomic patterns in body fluids present new opportunities for the development of novel, highly sensitive diagnostic tools for detection of cancer[4,5]. The urine proteome is known to provide detailed information for monitoring changes in the physiology of humans[5,6]. Urine collection is non-invasive and urine naturally occurring peptides (NOPs) have the advantage of being easily accessible without labour-intensive sample preparation[7]. These advantages make it potentially useful for a quick and reliable application in clinical settings. To prove the concept, it is possible to differentiate between different liver tumors (CLM, Hepatocellular Carcinoma (HCC), Hepatocellular Adenoma) and not only measure peptides involved in the process of general tumor growth in the liver, we conducted a pilot-study. In the current study we demonstrated we could discriminate between these liver tumors with the use of peptides found in urine (unpublished work, poster presentation ESMO 2010). In this study, we will focus on the identification and validation of urine NOPs to discriminate patients with CLM from healthy controls.

MATERIALS AND METHODS

Ethics Statement

The use of patient materials was approved by the medical ethical committee of Erasmus University Medical Center and written informed consent was obtained for all patients.

Patient selection

We selected patients with Colorectal Liver metastasis (CLM) and healthy kidney donors as controls. Inclusion criteria were; female gender, age above 18 years and written informed consent. The patients with CLM underwent liver resection and their diagnoses

were confirmed by the pathologist afterwards. Patients and controls were excluded if they were diagnosed with other malignancies or received prior chemotherapy.

A discovery set was formed of 23 patients that contained 11 patients with CLM and 12 controls. In addition, a validation set was formed with 26 patients, 13 with CLM, and 13 controls.

Sample collection

From patients with CLM, 100 ml urine was collected (midstream morning urine of sober patients). Fifty ml of urine was sent to the chemical laboratory at room temperature for determination of standard parameters (e.g. creatinine, total urine protein). Aliquots of 10 ml were made from the remaining 50 ml urine and stored within 4 hours from sample withdrawal at -80°C.

Sample preparation

Preparation of samples for proteomic analysis was performed as described previously[8] with some minor modifications. In brief, urine samples were thawed at room temperature and placed in a water bath for 30'at 30°C with regular mixing to dissolve the precipitate. At room temperature, 1.4 ml urine was centrifuged to remove remaining precipitate for 5' at 2000g. A volume of 1.2 ml of urine was diluted with 0.6 ml 3 M urea, 15 mM NH_4OH, 0.03% (w/v) SDS solution. From this mixture, 1.5 ml high molecular weight components were discarded using Centrisart ultracentrifugation columns (Sartorius, Goettingen, Germany) with a molecular cut-off limit of 20 kDa at a centrifugal force of 2500g for 10'. From this filtered sample 1.2 ml was applied to a PD-10 desalting column (GE Healthcare) equilibrated with 25 ml 0.01% NH_4OH and allowed to completely enter the filter bed. To improve the yield of natural occurring peptides (NOPs), 1.3 ml of equilibration buffer was applied to the filter bed as a first step and allowed to wash out by gravity flow. Subsequently, 2 ml of equilibration buffer was applied to the PD-10 column, the flow-through was collected, lyophilized, and stored at +4°C until further use. Prior to analysis by the nano-liquid chromatography/mass spectrometry (LC-MS), samples were suspended in 50 µl of HPLC-grade H_2O. For estimating the NOP content with the BCA assay (Pierce) 4 µL of sample was used. With the use of this information the sample was diluted to 0.8 µg peptide/µl to 0.1% trifluoroacetic acid/water for normalization.

Qualitative Mass spectrometry analysis (Orbitrap)

In the first part of the study, samples were measured with a nano liquid chromatography (LC) system (Thermo Fisher Scientific, Germaring, Germany) coupled on-line to a hybrid linear ion trap / Orbitrap mass spectrometer (LTQ-Orbitrap-XL, Thermo Fisher Scientific, Bremen, Germany). Samples were loaded onto a trap column (PepMap C18, 300 µm ID 5mm length, 5 µm particle size, 100 Å pore size; Thermo Fisher Scientific), washed and

desalted for 10 minutes using 0.1% trifluoroacetic acid (TFA) (in water) as loading solvent. Next, the trap column was switched in line with the analytical column (PepMap C18, 75 µm ID x 250mm, 3 µm particle and 100 Å pore size; Thermo Fisher Scientific) and peptides were eluted with the following binary gradient: starting with 100% solvent A, then from 0% to 25% solvent B in 60 min and from 25% to 50% solvent B in 30 min, where solvent A consisted of 2% acetonitrile and 0.1% formic acid (rest water), and solvent B consisted of 80% acetonitrile and 0.08% formic acid (rest water). All LC solvents were purchased at Biosolve, Valkenswaard, the Netherlands. Column flow rate was set at 300 nL/min. For electro-spray ionization (ESI), nano ESI emitters (New Objective, Woburn, MA, USA) were used and a spray voltage of 1.5 kV was applied. For Mass Spectrometry (MS) detection, a data-dependent acquisition method was used: high-resolution survey scan from 400 – 1800 Th. was detected in the Orbitrap (target of automatic gain control = 10 E6, resolution = 30,000 at 400 m/z, lock mass set to 445.120025 Th (protonated (Si(CH3)2O))6)[9]). On the basis of this full scan the five most intensive ions were consecutively isolated (AGC target set to 104 ions) and fragmented by collisional activated dissociation (applying 35% normalized collision energy) and detected in the ion trap. Precursor masses within a tolerance range of +/- 5 ppm that were selected once for MS/MS were excluded for MS/MS fragmentation for 3 minutes or until the precursor intensity fell below an S/N of 1.5 for more than five scans (early expiration). Orbitrap full scan spectra and ion trap MS/MS fragmentation spectra were acquired partially simultaneously.

Data analysis

The MS/MS data from the raw data files of each sample were converted into mgf files using Extract-MSN (part of XCalibur version 2.0.7, Thermo Fisher Scientific Inc.) and used to perform database searches using Mascot (version 2.2.06; Matrix Science Inc., London, UK) against the human subset of the Uniprot-Swissprot database (version 2011-3, human taxonomy, 20,287 entries). For database searches the following parameters were used: oxidation as a variable modification of methionine, hydroxylation as a variable modification of proline and lysine, maximal missed cleavage of 0, and "none" was selected as enzyme. A peptide mass tolerance of 10 ppm and a MS/MS mass tolerance of 0.5 Da were accepted. An ion score of 25 was used as a cut-off value. Subsequently, the raw data files were loaded into the software package Progenesis LCMS (Version 2.5; Nonlineair Dynamics Ltd, New Castle, UK) and aligned for retention time. Only features with a charge state of +2 to+8 were included for further analyses. Next, the results of the Mascot database search were imported into Progenesis, and an export file was created in which for each individual sample the abundances of the detected features were displayed. Abundance levels of masses identified multiple times in one sample were summed into single abundance values prior to statistical analysis. To eliminate

sporadic findings, identified masses not present in at least 3 samples of one group were subsequently excluded from further analysis.

Statistical analysis

Statistical analyses on the patient characteristics were conducted in the Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp). Categorical variables are presented as number (percentage). Continuous variables are presented as median (range), Categorical variables were compared, after testing for normality, with the χ^2 test; continuous variables were compared with the Independent *t*-test. A *p*-value <0.05 (two-sided) was considered significant.

Statistical analysis of the observations as obtained from mass spectrometry was performed using STATA (version 10, StataCorp, Texas, US). After testing for normality, using Shapiro-Wilks, univariate comparison between individuals from the groups was performed using Mann-Whitney *U*-test (parameter free) or unpaired t-test (parametric). This yielded many features (in raw abundance format) with significant outcome between the evaluated combinations. Subsequently, the identified features were analyzed using stepwise regression to construct a model containing a combination of those features with the highest sensitivity and specificity to distinguish the respective groups. This was done in combination with canonical linear discriminant analysis[10] to detect the various sensitivity and specificity details when the various models are applied.

Moreover, background evaluation was also performed in which models were applied with at random features and outcome to compare the acquired sensitivity and specificity with those as obtained after statistical analysis.

The discovery set was used to construct the model, consecutively the validation set, being independent from the discovery set, to check the acquired model.

Throughout the study, applying two-sided testing, a significance level of 0.01 was considered to be statistically relevant.

The discovery set was subjected to a univariate analysis in order to identify masses significantly different ($p<0.01$) between CLM patients and healthy controls that ought to be present in both discovery and validation. From the peptides with *p*-values <0.01 a best fit model was made in the discovery set and subsequently tested in the validation set to determine sensitivity and specificity.

Quantitative mass spectrometry analysis (SRM)

For the peptides selected based on the statistical analysis heavy labeled stable isotopes were ordered (Pepscan, Lelystad, The Netherlands). A selective reaction monitoring (SRM) method for the selected peptides was developed and optimized on a UPLC which

was online connected to a Xevo TQS mass spectrometer (Waters, Milford Massachusetts, USA).

The sample was trapped on a Symmetry C18 nanoACQUITY column (5 µm x 180 µm 20 mm) (Waters, Milford Massachusetts, USA) for 5 min and washed by a solution of 99% A and 1% B, solvent A 0.1% formic acid in water and solvent B is 0.1% formic acid in acetonitrile, with a flow of 8.00 µl/min. Followed by separation on an BEH 300 C18 column (1,7µm * 75µm * 200mm) (7 µm x 75 µm x 15 cm) with a flow of 0.3 µl/min and a gradient starting with 98.5 % A lowered in 30 min to 60 %. In 0.10 min it was further decreased to 20 % A and kept constant for 5 min following by an increase in 0.10 min, back to 98.5 % for 20 min. Ions were produced by a Z-spray nanoflow source under atmospheric pressure using a capillary voltage of 3.00 kV, cone voltage of 50 V and a source offset of 50 V. The source temperature was maintained at 70 °C. For every peptide 3 transitions were chosen differing in collision energy (table 1). Fragmentation is induced by collision dissociation with argon gas which is inserted with a flow rate of 0.15 ml/min. For the selection of the peptides for the final quantitative assay the following parameters were taken into account: No interference in used SRM transitions, co-elution of the peptide and internal standard (IS), linearity of response in measured concentration range, symmetry of peak shape and a signal intensity of at least 10 times the average observed background.

Table 1: The three transitions which have been developed for each peptide and the optimized collision energies.

Peptide	Parent mass (m/z)	Fragment (m/z)	Collision energy (V)
AGP P(-OH)GEAGK* P(-OH)GEQGV P(-OH)GDLGA P(-OH)GP	1088.51	527.28	37
		812.38	33
		1364.64	33
	1092.52	527.28	37
		812.38	33
		1372.66	33
K*GNSGEP(-OH)GAPGSK*GDTGAK*GEP(-OH)GPVG	786.04	442.23	29
		814.37	29
		957.94	25
	794.05	442.23	29
		822.38	29
		969.96	25

For each stable isotope labeled amino acid an extra mass of 8 Da is included.
m/z (mass-to-charge ratio); V (Voltage)

Skyline

The following parameters were taken into account for the selection of the peptides that were used for the SRM assay: no interference in used SRM transition, co-elution of the peptide and its internal standard (IS), linearity of response in measured concentration range and the peak intensity should be at least 10 times above the background level. The peak analysis was done with Skyline v1.3.0.3871 (MacCoss Lab, University of Washington, WA, United States of America). The results exported from Skyline were further analyzed with Microsoft Excel2007 (Redmond, WA, United States of America) and GraphPad Prism v5.00 (GraphPad Software, San Diego California USA) A ROC-curve was plotted by GraphPad Prism v5.00 (GraphPad Software, San Diego California USA). The cut-off value was chosen whereby both sensitivity and specificity were as high as possible.

RESULTS

Patient characteristics and clinical chemistry data of the urines are presented in Table 2. A significant difference in age (p=0.01) and Body Mass Index (BMI) (p=0.04) is observed. Kidney function and urine protein were comparable between both groups.

Table 2: Basic patient characteristics

Variable	CLM (n=24)	Healthy kidney donors (n=25)	p- value
Age[1,3]	64 (43-81)	57 (34-75)	0.01
BMI[1,3]	23.6 (18-36)	27.3 (20-35)	0.04
No. of lesions[1]	2 (1-7) [†]	-	-
Size largest lesion (cm)[1]	3 (1-10) [†]	-	-
Serum creatinine >115 μM[2,4]	1[†] (4%)	0	0.29
Urine protein >0.14 g/L[2,4]	3 (12.5%)	0	0.07

[†] 1 missing

CLM, Colorectal Liver Metastasis; BMI, Body Mass Index

[1] Data are presented as median with the range between brackets.

[2] Data are presented as numbers with the percentage between brackets.

Data were analyzed using a [3]Independent t-test or the [4] χ^2 test.

Qualitative Mass spectrometry analysis (Orbitrap)

Of the 28830 and 57276 masses detected in the discovery and validation set, respectively, 2426 (8.6%) and 3424 (5.8%) unique peptide sequences were identified. These naturally occurring peptides belong to 189 and 445 proteins, respectively. Of the unique peptides

identified 1386 (55%) derived from 26 different collagen proteins in the discovery set and 1303 (39%) from 34 in the validation set. Of all identified masses 80% of the abundant intensities is associated with collagen peptides. It occurred that same peptides were sequenced with one or more hydroxylated proline or lysine residues. Of all identified collagen peptides, 1702 peptides had 2 or more hydroxylations on either lysine or proline residues with a maximum of 11. These modified peptides are considered as unique peptides.

Statistical analysis and regression modeling

Univariate analysis of the discovery set revealed 40 collagen peptides that were significantly different (p<0.01) between the healthy individuals and patients with CLM.

A Multivariate analysis, using stepwise regression was applied in the discovery set to construct a relevant model using all identified peptides, showing significance between healthy individuals and CLM patients. To obtain a 100% sensitivity and 100% specificity a 17-collagen peptides model was identified (Canonical correlation:0.9911, Eigenvalue:55.64, F:16.37, p:0.003) (Table 3).

Table 3 : Sequences of the 17 Peptides used for the first model in the discovery set.

Mass (Da)	Sequence	Accesion code
2204.995	ADGQPGAKGEP(-OH)GDAGAKGDAGPP(-OH)GP	COL1A1
2175.011	**AGPP(-OH)GEAGKP(-OH)GEQGVP(-OH)GDLGAP(-OH)GP**	**COL1A1**
1927.909	DP(-OH)GETGEQGDRGIP(-OH)GHRG	COL1A1
1778.855	GAAGEP(-OH)GKAGERGVP(-OH)GPP(-OH)GA	COL1A1
2628.235	GLPGP(-OH)AGP(-OH)P(-OH)GEAGKP(-OH)GEQGVP(-OH)GDLGAP(-OH)GP	COL1A1
2561.128	GPP(-OH)GADGQP(-OH)GAP(-OH)GEP(-OH)GDAGAKGDAGP(-OH)PGP	COL1A1
2632.164	GPP(-OH)GADGQP(-OH)GAP(-OH)GEP(-OH)GDAGAKGDAGP(-OH)PGPA	COL1A1
2786.247	GPP(-OH)GADGQP(-OH)GAKGEP(-OH)GDAGAP(-OH)GDAGP(-OH)PGPAGP	COL1A1
2516.165	GPP(-OH)GKNGDDGEAGKP(-OH)GRP(-OH)GERGP(-OH)PGP	COL1A1
1734.781	GPP(-OH)GPP(-OH)GKNGDDGEAGKPG	COL1A1
1408.664	GPPGP(-OH)P(-OH)GP(-OH)PGPPGPPS	COL1A1
2371.086	P(-OH)GNSGEP(-OH)GAP(-OH)GSKGDTGAKGEP(-OH)GPVG	COL1A1
2355.098	**KGNSGEP(-OH)GAP(-OH)GSKGDTGAKGEP(-OH)GPVG**	**COL1A1**
1522.732	KP(-OH)GEQGVP(-OH)GDLGAP(-OH)GP	COL1A1
2989.483	NVGAP(-OH)GAKGARGSAGP(-OH)P(-OH)GATGFP(-OH)GAAGRVGPPGP(-OH)	COL1A1
2973.485	NVGAPGAP(-OH)GARGSAGPP(-OH)GATGFP(-OH)GAAGRVGP(-OH)PGP	COL1A1
2670.203	ERGEAGIP(-OH)GVP(-OH)GAP(-OH)GEDGKDGSP(-OH)GEP(-OH)GA	COL3A1

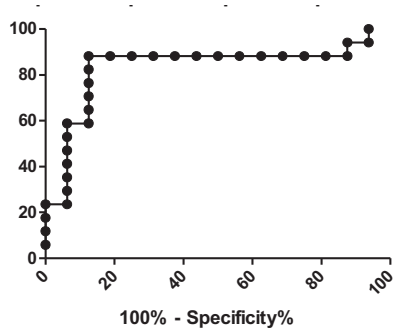
The amino acids which are underscored are hydroxylated. The two peptides which are written in bold are the finally selected peptides. (COL1A1 Collagen, type I, alpha 1)

Analysis of the samples from the validation set returned 58545 masses of which 3442 unique peptides could be identified. Out of these 3442 peptides, 1304 belonged to 34 different collagens.

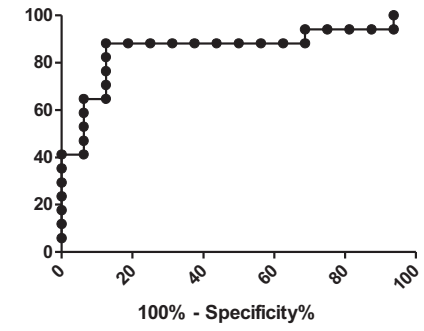
Within the validation set, of the original 40 peptides identified in the discovery set as having a significant difference in raw abundance between both groups, 7 peptides could be identified. It was decided to use all 7 peptides to model the outcome based on the results in the validation set. When these 7 peptides were applied in a discriminant analysis a sensitivity of 84.6% and a specificity of 92.3% were established (Canonical correlation:0.797, Eigenvalue:1.744, F:4.49, *p*:0.005).

Figure 1 ROC-curves of the selected peptides AGPP(-OH)GEAGKP(-OH)GEQGVP(-OH)GDLGAP(-OH)GP (A) and KGNSGEP(-OH)GAP(-OH)GSKGDTGAKGEP(-OH)GPVG

A



B



	Cut off	Sensitivity %	Specificity %	Area under the curve
AGPP(-OH)GEAGKP(-OH)GEQGVP(-OH)GDLGAP(-OH)GP	0.002794	88.24	87.50	0.8346
KGNSGEP(-OH)GAP(-OH)GSKGDTGAKGEP(-OH)GPVG	0.001011	88.24	87.50	0.8603

The peptides AGPP(-OH)GEAGK P(-OH)GEQGV P(-OH)GDLGA P(-OH)GP and KGNSGE P(-OH)GAPGSKGDTGAKGE P(-OH)GPVG were selected for further quantitative mass spectrometry analysis by selective reaction monitoring (SRM) based on a thorough evaluation on the criteria described in the materials and method section. The quantitative analysis resulted in a sensitivity of 88% and specificity of 89% (Figure 1).

These two peptides have been retested in the Orbitrap data showing a sensitivity and specificity of 72.7% and 100% in the discovery set and 69.2% and 84.6% in the validation set.

DISCUSSION

Patients

For this study healthy kidney donors have been selected as a control group. These controls were selected because living kidney donors are examined intensively to exclude any disease. Because this specific control group was chosen, which were not matched with our CLM-patients, a difference in patient characteristics in BMI (p=0.04) and age (p=0.01) was found. Only women were selected because the first pilot study we have performed included different solid liver tumors including HCA, a benign liver tumor which occurs very rarely in men[11].

In this study, a collagen profile has been discovered and validated in patients who were diagnosed with colorectal liver metastasis at the time of sampling. To identify the prognostic value of our profile, we will continue sampling during the regular follow-up in patients who underwent surgery because of CRC. Thereby patient variation presumably can be diminished and an even better sensitivity and specificity can be expected. The short coming of this study is the small number of patients. However, we believe we describe a very innovative concept for the detection of colorectal liver metastasis. Furthermore, the detection of different amounts of hydroxylated collagen type 1 may proof to be very valuable for clinical use and, in addition, it could contribute to the understanding of the liver seeding and homing of colorectal metastasis. Larger experiments need to be performed to demonstrate the efficacy of this approach.

Peptide selection

In this study, only two stable isotope labelled peptides were technically suitable in our final analysis (table 3). Mischak *et al*. already showed data generated by different proteomics technologies are not always comparable[12]. Although the result could possibly be improved by taking more significant different peptides, this study already shows the new possibilities to use urine proteomic analysis to detect CLM.

Collagen

The two peptides identified in this study are part of collagen type 1. Collagens are macromolecular molecules which are eliminated due to secretion by the kidney. Type I collagen is the most abundant in stroma which is composed of the extracellular matrix (ECM). In the adult liver the ECM is mostly composed of collagen type 1 and fibronectin[13-16]. The structure of the ECM is not static, it remodels constantly as a consequence of development and disease[17]. This remodelling is a result of multiple processes that vary according to the initiating stimulus. The protein components of the ECM are cleaved by metalloproteinases (MMPs) and they seem to play a dominant role in this process of remodelling[18]. The remodelling of the ECM is an essential event before invasion of neoplastic cells into the stromal tissue and could explain our findings in the urine of patients with CLM. The different expressions of type 1 collagen were also described in the stromal composition of tissue samples from CRC and CLM[19]. The combination of these results provides evidence that type 1 collagen has a role in CLM. However further research is needed to support these findings. Previously our research group identified tumour specific collagen-like peptides which are located in the blood vessels of brain tumours. These proteins are expressed in tumour blood vessels, but not in blood vessels of healthy brain tissue[20].

Hydroxylation

Hydroxylation of peptides provides further stabilization or, depending on the location of the hydroxylation, the opposite, namely instability. Normally the hydroxylation in collagen sequences happens at the terminal residue in Gly–Pro–Pro repeats[21]. The final classifier existed of two hydroxylated collagen peptides with a sensitivity of 88%, a specificity of 88%. We hypothesize that in the liver the post-translational modification related to hydroxylation in collagen is altered due to the cancer cell invasion.

Although this study is based on relatively small numbers, urine proteomic analysis revealed two very promising peptides AGPP(-OH)GEAGK P(-OH)GEQGV P(-OH)GDLGA P(-OH)GP and KGNSGE P(-OH)GAPGSKGDTGAKGE P(-OH)GPVG, which are both part of collagen 1, to detect CLM in a non-invasive manner.

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Chapter 5

Hydroxylated collagen peptide in urine as biomarker for detecting colorectal liver metastases

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ABSTRACT

The clinical efficacy of carcinoembryonic antigen (CEA) as a marker of colorectal liver metastasis is limited, motivating a search for new biomarkers. Recently, urine proteomic analysis revealed AGPP(-OH)GEAGKP(-OH)GEQGVP(-OH)GDLGAP(-OH)GP (AGP), a promising peptide for this application. This study aimed to determine whether combining urine AGP testing with serum CEA analyses improves the sensitivity of detecting colorectal liver metastases. Urine samples from 100 patients with CLM were collected prospectively and compared to three control groups: healthy kidney donors, patients who were relapse-free for 24 months after curative CLM surgery, and primary colorectal cancer patients. A stable isotope labeled peptide standard was used to quantify the abundance of AGP in urine samples by selective reaction monitoring. Combined testing of urine AGP levels and serum CEA levels revealed a significantly increased sensitivity compared to CEA alone (85% *vs*. 68%, $p<0.001$; specificity 84% and 91%, respectively). No correlation was found between CEA and AGP-positive test results within individual patients ($r^2 = 0.08$). Urine AGP testing was negative in the three control groups. These results indicate that collagen-derived urine AGP peptide with a specific hydroxylation pattern combined with serum CEA levels may significantly improve the detection of colorectal liver metastases in patients at risk.

INTRODUCTION

Colorectal cancer (CRC) represents one of the most common malignant diseases, with 1.2 million new cases a year worldwide[1]. Even after curative surgical resection of the primary tumor, 25–40% of CRC patients will develop colorectal liver metastases (CLM)[2-5]. Follow-up aims to detect metastases at an early stage, offering additional treatment and survival benefit[6]. Early detection of CLM leads to better results with the application of surgery or local ablation[2]. Although surgery may offer the best outcome, 80% of all patients with CLM are not considered candidates for resection due to advancement of the disease beyond curative treatment options[7]. Therefore, the ASCO guidelines recommend an intensive follow-up every 2–3 months during the first 2 years after surgery[8].

Although follow-up protocols for patients who undergo curative resection for CRC differ worldwide, all advise ultrasound (US), computed tomography (CT), and/or carcinoembryonic antigen (CEA) testing[5]. Ultrasound has a sensitivity of approximately 57% and a specificity of 91%. Computed tomography performs slightly better (sensitivity approximately 68% and specificity 96%)[9]. A positive CEA test has a sensitivity of ~64% for detecting CLM[10]. The low sensitivity of CEA is due to the fact that not all colorectal tumors and their metastases produce CEA, leading to false negatives[10,11]. However, CEA has a high specificity, as it is rarely elevated in the absence of CRC[12].

Recently, we performed urine proteome analysis and demonstrated two promising peptides to detect colorectal liver metastases, both being part of collagen type 1(I): AGPP(-OH)GEAGKP(-OH)GEQGVP(-OH)GDLGAP(-OH)GP (AGP) and KGNSGEP(-OH) GAPGSKGDTGAKGEP(-OH)GPVG (KGN). These peptides had a sensitivity of approximately 88% and a specificity of 88%. When AGP and KGN were combined, they had a sensitivity of 85% and a specificity of 92% in a discovery setting[13]. As AGP and KGN showed a strong correlation with a better performance for AGP we continued our studies with urine AGP analysis.

In this study, we determined the additional value of urine AGP screening in addition to serum CEA levels to identify patients with CLM.

MATERIALS AND METHODS

Ethics Statement

The use of patient materials was approved by the medical ethics committee of Erasmus MC (MEC-2008-062). Written informed consent was obtained from all patients and controls for our prospective observational case-control study.

Sample vials were de-identified by numerical coding; only the people involved had access to patient information.

Patient Selection

A total of 100 adult patients (age ≥18 years) with radiologically confirmed CLM who were planned to undergo surgical resection of metastatic liver lesion(s) (ICD 10 C18-C19) were prospectively selected. Patients with the primary tumor in situ or concomitant malignant diseases were excluded. All patients provided written informed consent. The diagnosis of CLM was confirmed by the pathologist in the resection specimens of all patients.

Three groups of control subjects were used. The first group of controls consisted of 100 healthy kidney donors (HKDs) who had a complete blood examination and abdominal CT imaging prior to donation and were considered healthy. The second group of controls consisted of 20 patients who underwent liver surgery for CLM and were relapse-free for at least 24 months (relapse-free controls, RFCs). The third group of controls included 18 patients with primary CRC in situ without CLM as demonstrated by CT –imaging (primary colorectal cancer controls, PCCs). For all patients, the diagnosis of CRC was confirmed by the pathologist. None of the patients received neoadjuvant chemotherapy or radiotherapy.

Study Design

Serum and urine samples were prospectively collected in three teaching hospitals in the city of Rotterdam, the Netherlands. Urine sampling was designed to mimic the clinical setting as much as possible. Urine was collected randomly during the day and the time to aliquoting and freezing the urine varied depending on the routine schedule of the hospital, but it was always within 4 hours of withdrawal. Clinical data were retrieved from (electronic) medical records, including, age, gender, body mass index (BMI), number of lesions, size of the largest lesion, and serum creatinine.

Specimen Characteristics

Midstream urine samples (50 ml) were collected from all patients and controls the day before surgery, or in case of the RFC group at the time of inclusion. Samples were stored as 10 ml aliquots in 15 ml BD Falcon tubes at -80°C within 4 hours of sample withdrawal. No additives were added prior to sample processing. Freeze-thaw cycles were kept to a minimum, with a maximum of two cycles per sample prior to sample processing. CEA measurements were part of the standard follow-up of patients with CRC and were extracted from the (electronic) patient files.

An additional serum sample was taken from the controls to determine serum CEA levels using the Elecsys CEA quantitative electrochemiluminescence immunoassay (Roche, Switzerland).

Chemicals

UHPLC solvents were obtained from Biosolve (Valkenswaard, the Netherlands) and all other chemicals from Sigma Aldrich (Zwijndrecht, the Netherlands). The stable isotope-labeled peptide was obtained from Pepscan (Lelystad, the Netherlands). The hydroxylation pattern of the stable isotope-labeled peptide on MS^2 spectra was compared to that of AGP. Both spectra had a high overlap and similar hydroxylation pattern that confirmed the hydroxylation pattern of the identified human peptide (Figure 1).

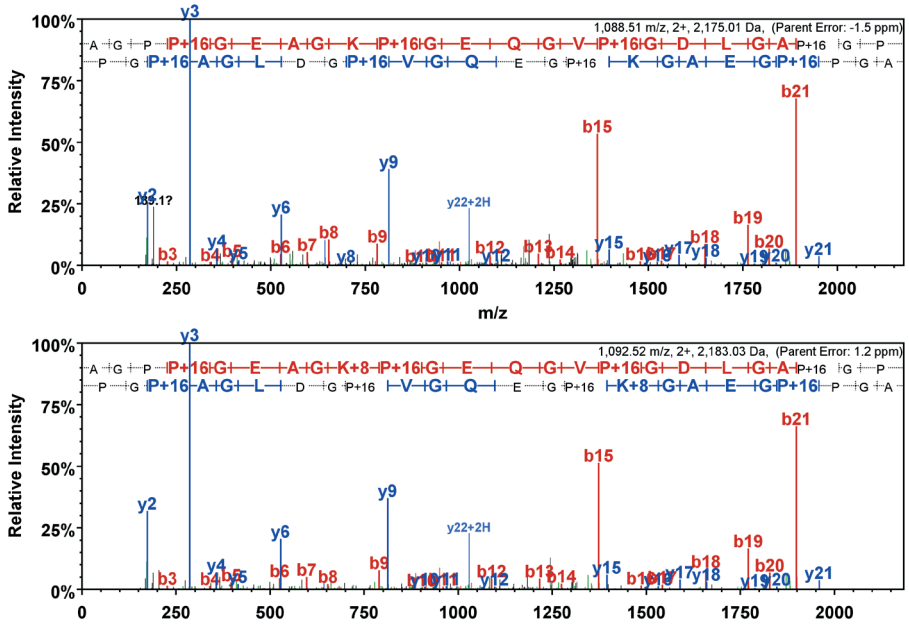

Figure 1 The hydroxylation pattern of AGP was confirmed by MS^2. The high resolution spectra of endogenous (top) and stable isotope-labeled peptides (bottom) were compared and found to be identical

Sample Preparation

An automated sample preparation method that was cross-validated with the previous method published by Bröker *et al*. was applied (data not shown)[13]. Unless otherwise stated, samples were processed at room temperature. Prior to sample preparation the urine samples were thawed, first at room temperature for 1 h, and then in a 37°C water bath for 15 min. The samples were then vortexed for 5 s. Subsequently, 1.4 µl of a 5 µM internal standard and 0.5 ml of urine were transferred to a 96 deep well plate. The

samples were frozen at -32°C, lyophilized overnight, and dissolved by adding 200 µl of 10 M urea. Samples were shaken on a plate shaker (Eppendorf) for 30 s at 800 rpm and then centrifuged for 5 min at 2500 g to precipitate insoluble particles. One hundred microliters of sample was transferred from each well to a 96-well plate, which was sealed with adhesive aluminum foil to prevent evaporation and contamination of the samples. The samples (40 µl) were separated on an mRP C-18 Hi-Recovery Protein Column (4.6 x 50 mm) (Agilent, Amstelveen, the Netherlands) installed in an Ultimate 3000 (Dionex, Amsterdam, the Netherlands). Solvents A and B were 0.1% TFA in water and 0.1% TFA in acetonitrile, respectively, with a flow rate of 750 µl/min. The column was kept at a constant temperature of 80°C. The gradient was started with 100% solvent A and reduced after 4 min in a 0.4 min step to 75% solvent A. At 6.0 min, solvent A was increased in 0.1 min to 80% and then kept constant for 2.4 min, followed by a decrease in solvent A over 24 s to 30%. It was then kept constant for 1.5 min. At 10.5 min, solvent A was further decreased over 15 s to 5%. Finally, at 13.3 min the column was equilibrated for 6.45 min with 100% solvent A.

A portion of the flow was collected in a 96 deep well plate; the fractionation was started at 4.9 min and stopped at 6.5 min. The solvent was evaporated using a SpeedVac. The samples were then dissolved in 50 µl of 0.1% TFA. Each sample (5 µl) was injected and analyzed in a nanoAcquity Xevo-TQS mass spectrometer (Waters, Milford, Massachusetts, USA). The samples were measured in a randomized order. Urine AGP levels were expressed as the analyte/internal standard (IS) ratio.

Quantitative Mass Spectrometry

The liquid chromatography and mass spectrometry settings were similar to those published by Bröker *et al*.[13].

A selective reaction monitoring (SRM) method was developed and optimized on a Waters nanoAcquity Ultra Performance LC connected online to a Xevo TQS mass spectrometer (Waters, Milford, Massachusetts, USA). The sample was trapped on a Symmetry C18 nanoAcquity column (5 mm × 180 µm × 20 mm) (Waters, Milford, Massachusetts, USA) for 5 min and washed with a solution of 99% A and 1% B (solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid in acetonitrile) at a flow rate of 8.00 ml/min, followed by separation on an Acclaim PepMap100 C18 3 µm column (75 µm × 150 mm) at a flow rate of 0.3 ml/min and gradient starting with 98.5% solvent A reduced over 30 min to 60%. In 0.10 min it was further decreased to 20% solvent A and kept constant for 5 min, followed by an increase over 0.10 min to 98.5% and kept constant for 20 min. Ions were produced by a Z-spray nanoflow source under atmospheric pressure using a capillary voltage of 3.00 kV, cone voltage of 50 V, and a source offset of 50 V. The source temperature was maintained at 70°C. For AGP and the stable isotope-labeled peptide, three transitions with different collision energies were chosen (Table 1). Fragmentation

Table 1: The three transitions and collision energies used for detection of AGP and corresponding stable isotope-labeled internal standard. *, stable isotope-labeled lysine (K) (+8Da).

Peptide	**Parent mass *(m/z)***	**Fragment *(m/z)***	**Collision energy (V)**
AGP P(-OH)GEAGK P(-OH) GEQGV P(-OH)GDL (z=+2)	1088.51	527.28 (y6)	37
		812.38 (y9)	33
		1364.64 (b15)	33
AGP P(-OH)GEAGK* P(-OH)GEQGV P(-OH) GDL(z=+2)	1092.52	527.28 (y6)	37
		812.38 (y9)	33
		1372.66 (b15)	33

was induced by collision dissociation with argon gas inserted with a flow rate of 0.15 ml/min. The following parameters were taken into account for the selection of AGP for the final quantitative assay: no interference in SRM transitions, co-elution of the peptide and IS, linearity of response in measured concentration range, symmetry of peak shape, and a signal intensity at least 10-times the average observed background. A chromatogram of a sample with the lowest AGP level (ratio = 0.170) is shown in Fig. 2a; peaks are measured properly with a high signal to noise ratio and a symmetrical peak shape. The selected transitions were relatively free of interference.

Power Calculation

In a previous pilot study we demonstrated a sensitivity of 85% and specificity of 92% for the combination of two collagen urine peptides, AGP and KGN, in urine[13]. With the availability of urine samples from 100 patients with CLM followed over a 3-year period, the 95% confidence interval (CI) and estimated the sensitivity and specificity was calculated. The CIs for the estimated sensitivity and specificity of 70, 80, and 90% were 60–79%, 71–87%, and 82–95%, respectively. We judged these CIs to be sufficiently small and used a control group of 100 normal subjects (healthy living kidney donors; HKDs).

Quality Controls

One quality control (QC) was inserted per row of the 96-well plates. A total of 18 QC samples were measured with an average ratio of 0.232 and 14.6% CV. The QC AGP level was in the lower region of the measured concentrations, which are generally more receptive to variation; therefore, the CV indicated good reproducibility.

Statistical Analysis

The peak analysis was performed using Skyline v1.4.0.4421 (MacCoss Lab, University of Washington, WA, USA). Skyline is an open source, freely available application which can be used to refine targeted methods for large-scale quantative mass spectrometry studies in life sciences. Next, ROC-curves were used to generate cut-off values and to determine sensitivity and specifity. These analyses were performed using Microsoft Excel 2007 (Redmond, WA, USA) and GraphPad Prism v5.00 (GraphPad Software, San Diego, CA, USA).

Differences in the basic patient characteristics and treatment outcomes were assessed using the unpaired *t*-test and χ^2 test. Correlations between markers were assessed using the Pearson correlation coefficient. A p-value < 0.05 was considered significant. Missing data were supplemented using multiple imputation[14]. The basic characteristics were analyzed using SPSS (Version 21.0. Armonk, NY: IBM Corp.).

To investigate the capabilities of CEA and AGP to discriminate between controls and patients with CLM the distinctiveness of serum CEA and urine AGP levels were investigated separately (Figure 2a and b).

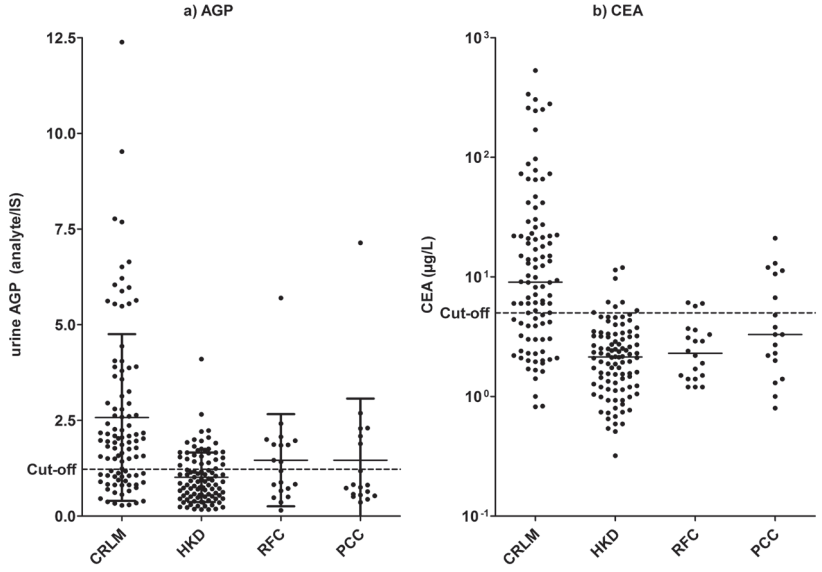

Figure 2a en b Distribution of data points based on a) the urine AGP ratios and b) serum CEA levels. AGP cut-off = 1.223, CEA cut-off = 5 µg/L. HKD = healthy kidney donors; RFC = relapse-free controls; PCC=primary colorectal cancer controls.

Next, a multivariate logistic regression analysis was performed. Potential non-linear effects of CEA and AGP in the log odds of having CLM were looked for using restricted cubic splines with three internal knots. The p-values for non-linearities were calculated based on the Wald test. In a second stage, the discriminative ability of the fitted model quantified by the RN^2 index of NagelKerke and Somers' Dxy rank correlation between predicted probabilities and observed responses (in other words, the model's ability to distinguish patients with CLM from controls) was investigated[15]. For both measures, values close to one indicate good predictive performance[16]. These measures were validated to account for possible over-fitting using the Bootstrap method taking 500 re-samples. Results and conclusions are based on the corrected (i.e., validated) RN^2 and Dxy indexes. The regression analysis and model fitting were performed in the R programming language (version 3.1.3).

Since the group sizes were unequal, the Tukey's contrasts test was used to test for significant differences between the four groups. Tukey's contrasts test was also performed in the R programming language (version 3.1.3.).

RESULTS

Basic Characteristics

The basic characteristics of both patients and controls are presented in Table 2. The median age and percentage of males was significantly different between the four groups (both $p<0.001$). No difference in BMI was found between the groups ($p=0.541$). The median number of liver lesions was 2 (IQR 1–4), with a median diameter of 2 cm (IQR 1.8–4.0 cm). The serum creatinine level was > 115 µmol/L for several subjects (CLM $n=4$, HKD $n=0$, RFC $n=2$, PCC $n=1$), indicating impaired renal function.

Table 2: Basic characteristics of patients with colorectal liver metastases (CLM) and controls

	CLM (n=98)	HKD (n=100)	RFC (n=20)	PCC (n=18)	P-value ANOVA
Age	64 (57-70)	52 (43-63)	72 (63-81)	73 (69-81)	<0.001
Gender, male	71 (72%)	37 (37%)	11 (55%)	13 (72%)	<0.001
BMI	26 (25-28)	25 (23-28)	27 (23-29)	26 (23-28)	0.541
No. of lesions	2 (1-4)	-	-	-	-
Size of largest lesion, cm	2.7 (1.8-4.0)	-	-	-	-
Serum creatinine >115 µM/L	4 (4%)	0 (0%)	2 (10%)	1 (6%)	0.062

HKD = healthy kidney donors; RFC = relapse-free controls; PCC = primary colorectal cancer controls; BMI = body mass index.
Data are presented as a median with the interquartile range (25th – 75th percentile) or n(%).

CEA and AGP

A relatively large proportion of patients in the CLM group (34%) had serum CEA levels below the cut-off value (5 ng/ml, Table 3). In the control groups, several subjects had elevated CEA levels (HKD *n*=8, RFC *n*=3, PCC *n*=6). As shown in Table 4, the serum CEA levels in the CLM group significantly differed from the serum CEA levels in all control groups individually, whereas the controls did not significantly differ from each other. The sensitivity and specificity was 66% (95% CI 56–76%) and 92% (95% CI 85–96%), respectively, when comparing the CLM and HKD groups.

Table 3: Serum CEA levels and urine AGP levels in patients with colorectal liver metastases (CLM) and controls

	CLM (n=98)	HKD (n=100)	RFC (n=20)	PCC (n=18)
Serum CEA (ng/ml)	9.05 (3.90-22.93)	2.14 (1.22-3.21)	2.3 (1.48-3.38)	3.3 (2.05-9.63)
Serum CEA >5 ng/ml	65 (66%)	8 (8%)	3 (17%)	6 (33%)
Urine AGP (analyte/IS)	1.96 (1.06-3.23)	0.9 (0.53-1.44)	1.3 (0.71-1.9)	0.78 (0.56-2.04)
Urine AGP >1.223 (analyte/IS)	66 (67%)	27 (27%)	10 (50%)	6 (33%)
Mult. Var.	10.38 (1.73-158.42)	0.19 (0.08-0.47)	0.32 (0.19-0.89)	0.44 (0.12-2.93)
Mult. Var. >0.6278	83 (85%)	16 (16%)	6 (33%)	8 (44%)

HKD = healthy kidney donors; RFC = relapse-free controls; PCC = primary colorectal cancer controls; IS = internal standard; CEA: carcinoembryonic antigen; AGP: (AGPP(-OH)GEAGKP(-OH)GDLGAP(-OH)GP).
Urine AGP ratios were used in calculations. The lowest and highest quartile (ratio of 0.53 – 3.23) represents a concentration range of 7.4 – 45.2 nmol/L (16 - 98 µg/L).
Data are presented as the median with the interquartile range (25th – 75th percentile) or n(%).

Table 4: Univariate and multivariate group comparisons using regression analysis

Group comparison	AGP p-value	CEA p-value	Mult Var p-value
CLM - CRC	0.0283	<0.001	<0.001
HKD - CRC	0.6713	0.127	0.0984
RFC - CRC	1.0000	0.572	0.798
HKD - CLM	<0.001	<0.001	<0.001
RFC - CLM	0.0197	<0.001	<0.001
RFC - HKD	0.6397	0.932	0.615

CLM = colorectal liver metastasis; HKD = healthy kidney donors; RFC = relapse-free controls; PCC = primary colorectal cancer controls.
P-values were calculated using Tukey's contrasts.

Urine AGP levels were measured in all CLM samples except for two in which no signal was observed. In the CLM group, 33% of the urine AGP levels were below the cut-off value of 1.223, which was the optimal cut-off point calculated with an ROC curve (Table 2). In the control groups, several subjects had elevated urine AGP levels (HKD *n*=27, RFC *n*=10, PCC *n*=6). Urine AGP levels in the CLM group significantly differed from the urine AGP levels in

all control groups individually, but the controls did not significantly differ from each other (Table 3). The AGP test had a sensitivity and specificity of 68% (95% CI 58–77%) and 69% (95% CI 59–78%), respectively. No significant correlations were found between AGP and the size of the liver lesion, the number of lesions, or liver enzyme values for alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma glutamyl transferase (γ-GT).

Serum CEA levels and urine AGP levels did not correlate ($r^2 = 0.08$) and, therefore, were complementary.

Multivariate Logistic Regression Model

A multivariate logistic regression model was created:

$$Odds\ of\ being\ sick = e^{3.9090+1.1213\ AGP\ +\ 1.622\ \ln(CEA)}$$

Based on this model, the combined value of serum CEA and urine AGP was significantly different for the CLM group compared to all individually tested control groups, whereas the combined values in the controls groups did not significantly differ (Table 3). The final model produced with an AUC of 0.9139 (95% CI 0.8745-0.9532) resulted in a sensitivity of 85% (95% CI 76-91%) and specificity of 84% (95% CI 75-91%) with a corresponding optimal cut-off value of 0.6278 (Figure 3).

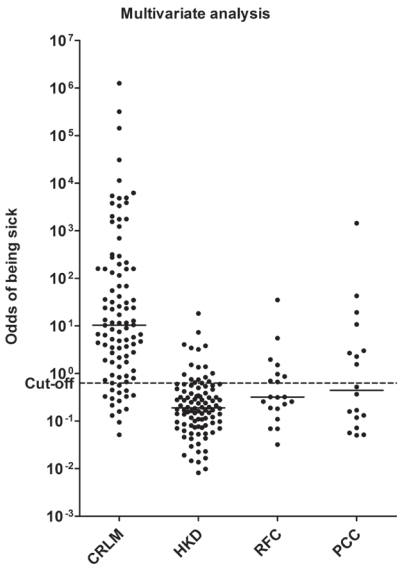

Figure 3 Distribution of data points based on the final multivariate regression analysis combining serum CEA and urine AGP levels. HKD = healthy kidney donors; RFC = relapse-free controls; PCC=primary colorectal cancer controls

DISCUSSION

The current study demonstrates that collagen-derived peptide AGP has a very specific hydroxylation pattern that can be reliably measured in urine using mass spectrometry. When combined with serum CEA levels, urine AGP demonstrates to be a promising biomarker with a sensitivity of 85% (95% CI 76-91%) and specificity of 84% (95% CI 75-91%).

Clinical proteomics using mass spectrometry has yielded early and positive results in different diseases[17]. These results have the potential to detect patients with a specific disease but need to be confirmed in large-scale studies[18]. Large-scale validation is essential for assessing the value of biomarkers, as large independent validation studies have often shown less promising results than small discovery sets[17,19]. Due to the cost and time required for prospective sample collection, preliminary results are often presented. In a small discovery study consisting of 24 patients, we reported a sensitivity and specificity of 88% for AGP[13]. In the current study with 100 patients, the sensitivity decreased to 68% for AGP alone. This decrease may be attributed to the increased variation in patients and sampling conditions, since both males and females were included, urine was collected randomly during the day and the time to aliquoting and freezing the urine varied depending on the routine schedule of the hospital. However, even with these more variable conditions, the sensitivity of AGP combined with CEA clearly exceeded that of CEA alone (85% vs. 68%), which is similar to the sensitivity of CEA combined with liver imaging[5].

This study focused on AGP, a naturally occurring hydroxylated peptide that is part of collagen type α1(I)[13]. Collagens are the most abundant proteins in the animal kingdom. In the human body, 80-90% of the total collagen is collagen type I, II, or III[20]. In our study, not the amino acid sequence, but more the hydroxylation pattern for AGP found appears to be very specific since the chance that this specification hydroxylation occurs at the specific positions can be estimated to be 0.00072% based on a chance process described by Rapaka *et al* [21]. The location from which the AGP peptide with the specific hydroxylation pattern found in the urine of patients with CLM is derived remains unclear. However, it is tempting to suggest that it is derived from the liver, either from the metastasis or the metastasis surrounding tissue. One may reason that it is not likely that AGP originates from more central, hypoxic regions of the tumor as the lack of oxygen inhibits the hydroxylation of proline by prolyl 4-hydroxylase. This assumption is supported by the previously described decrease in hydroxylation of hypoxia-induced factors inhibiting degradation in hypoxic regions[22] and suggests the formation of collagen with a limited number of hydroxylations in hypoxic tumor regions, whereas AGP is fully hydroxylized. A more plausible origin may be the activity at the invasion front, including increased tissue remodeling and production of collagen, thus enabling tumor progression with enhanced production of matrix metalloproteinase (reference) and an

increase in urine AGP levels with a specific hydroxylation pattern, as observed in this study.

CEA, the standard biomarker used for CLM, is known for its high specificity. Ten subjects in the control groups (7%) had serum CEA levels > 5 ng/ml. Other factors that have been linked to an increase in CEA include smoking, the use of paroxetine (a selective serotonin reuptake inhibitor, SSRI), metabolic syndrome, and alcoholic liver disease[23-28]. Of the 10 subjects, four were medium to heavy smokers (15-25 cigarettes per day), three used an SSRI at the time of CEA determination, one was diagnosed with metabolic syndrome, and one was diagnosed with alcoholic liver disease, leaving only one control subject with an unexplained elevation of CEA.

From a clinical perspective, the main goal of our study was to increase the sensitivity of CEA to more accurately identify CLM in patients with a medical history of resected CRC. When comparing CEA alone and combined with the urine peptide, the sensitivity increased from 68% to 85% and specificity decreased from 91% to 84%.

Further research is needed to evaluate the potential of using the combined biomarkers to detect CLM at an earlier stage, possibly resulting in more effective interventions. Longitudinal sampling is expected to be of value and may improve the sensitivity, as an increase within one patient can be observed. More research should be performed in patients with CLM who have a false negative AGP. Whether these false negative patients are positive after multiple testing at various time points should be investigated. To answer these questions and to validate the added value of the AGP peptide, a large follow-up study should be performed in which urine AGP levels are determined in addition to the regular follow-up tests (CEA and US and/or CT). Ultimately, a test may be constructed in which the urine AGP test and serum CEA test are combined and routine imaging is needed less frequently.

In conclusion, the collagen-derived urine AGP peptide with a very specific hydroxylation pattern can be measured reliably using mass spectrometry and may be a promising biomarker to reliably identify CLM in combination with serum CEA levels.

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Chapter 6

Anatomical versus Non-anatomical resection of Colorectal Liver Metastases: Is there a difference?

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ABSTRACT

Background: The increased use of neoadjuvant chemotherapy and minimally invasive therapies for recurrence in patients with colorectal liver metastases (CLM), makes a surgical strategy to save as much liver volume as possible pivotal. In this study, we determined the difference in morbidity and mortality and the patterns of recurrence and survival in patients with CLM treated with anatomical (AR) and nonanatomical liver resection (NAR).

Methods: From January 2000 to June 2008, patients with CLM who underwent a resection were included and divided into two groups: patients who underwent AR and patients who underwent NAR. Patients who underwent simultaneous radiofrequency ablation in addition to surgery and patients with extrahepatic metastasis were excluded. Patient, tumor and treatment data as well as disease-free survival (DFS) and overall survival (OS) were compared.

Results: Eighty-eight patients (44%) received AR and 113 patients (56%) underwent NAR. NAR were performed for significant smaller metastases (3cm *vs*. 4cm, $p<0.001$). The Clinical Risk Score did not differ between the groups. After NAR, patients received significantly less blood transfusions (20% *vs*. 36%, $p=0.012$) and the hospital stay was significantly shorter (7 *vs*. 8 days, $p<0.001$). There were no significant differences in complications, positive resection margins or recurrence. For the total study group, estimated 5-year DFS and OS was 31% and 44%, respectively, with no difference between the groups.

Conclusions: Our study resulted in no significant difference in morbidity, mortality, recurrence rate, or survival according to resection type. NAR can be used as a save procedure to preserve liver parenchyma.

INTRODUCTION

Colorectal cancer is the most common gastrointestinal malignancy worldwide, affecting nearly one million people each year[1]. Half of these patients have or will develop hepatic metastases at some point during their life. Liver resection is considered to be the best treatment for colorectal liver metastases (CLM) with 5-year survival rates up to 60% in highly selected patients[2]. Until recently, only 10-20% of patients were considered suitable for attempted curative resection[3,4]. Due to improvements in surgical techniques, the acceptance of resection margins smaller than 1cm[5,6], the introduction of more effective systemic chemotherapeutics[7,8], the use of portal vein embolisation (VPE)[9,10], the addition of radio frequency ablation (RFA)[11,12] and stereotactic body radiation (STBR)[13] to surgery, more patients are eligible for liver surgery. Moreover, the indications for liver resection have expanded over the past decade and there are only few limitations left, which include unresectable extrahepatic disease and insufficient future remnant liver. The question nowadays has shifted from "what can be resected" to "what will be left".

During this period a change in surgical approach can be observed by an increase of non-anatomic resections[14]. A non-anatomical resection maximizes the amount of residual liver parenchyma which is important, in particular for patients who received neoadjuvant chemotherapy. While chemotherapy increases resectabilty, it is associated with hepatic changes, which might increase the risk of progressive hepatic failure and death after resection[15,16]. Moreover, in case of intra-hepatic recurrences after partial liver resection in patients with CLM, a sufficient liver residual can offer the opportunity for local treatment[17].

Although anatomical hepatic resection has been reported to improve patient survival in hepatocellular carcinoma (HCC)[18-20], the literature about CLM is conflicting.

The purpose of this study was to investigate the influence of a non-anatomical resection (NAR) compared to an anatomical resection (AR) on morbidity, mortality, margin positivity, disease free survival (DFS) and overall survival (OS).

METHODS

All patients undergoing partial hepatic resection for CLM at the Erasmus Medical Center from January 2000 to June 2008 were evaluated for inclusion in this study.

Patients who underwent simultaneous AR and NAR or received additional RFA in addition to surgery as well as patients with extra hepatic metastasis were excluded.

Patients were divided into two groups: patients who underwent an AR and patients who underwent a NAR. An AR was defined as resection of two or more hepatic segments as described by Couinaud[21]. This includes, bisegmentectomy, (extended) right

hemihepatectomy, (extended) left hemihepatectomy or a combination of these[22]. NAR was defined as resection of the CLM including a rim of microscopically normal tissue. The choice of resection type was made in a multidisciplinary hepatobiliary working group, based on tumor number, location and patient status.

Information collected included demographic details, primary tumor stage (TNM-classification), maximum size, number and distribution of liver metastases on CT, plasma carcinoembryonic antigen (CEA) levels, neoadjuvant chemotherapy, Clinical Risk Score (CRS)[23], type of liver surgery, transfusion data, overall duration of hospital stay, perioperative complications, radicality, site and treatment of recurrence.

OS and DFS were calculated from the date of liver resection. Complications or death occurring either within 30 days or before discharge were considered perioperative. We defined a positive surgical margin as the presence of vital tumor along the line of transection.

After partial hepatic resection, patients routinely underwent a physical examination and determination of CEA-level, abdominal/chest CT or ultrasonography every 4 months for the first year, every six months the second year and once a year thereafter.

Statistical analyses were conducted using SPSS (version 15, SPSS Inc., Chicago USA). Categorical variables are presented as number (percentage). Continuous variables are presented as median (range). Categorical variables were compared with the χ^2 test; continuous variables were compared with the Mann-Whitney-*U* test. Actuarial survival was calculated using the Kaplan-Meier method from the date of resection of CLM, and differences in survival were examined using the log-rank test. $p < 0.05$ (two-sided) was considered significant.

RESULTS

Clinicopathological variables

Between January 2000 and June 2008, 308 patients underwent a partial hepatic resection for CLM; 201 patients met the study inclusion criteria, including 126 men (63%) and 75 women (37%). The median age was 65 years (range, 30-86). The primary tumor was located in the colon in 114 patients (57%) and rectum in 87 patients (43%). After resection of the initial tumor, positive lymph nodes were present in 114 patients (57%); synchronous liver metastases were identified in 78 patients (39%). The median disease free interval for the remaining 123 patients was 20 months (range, 4-193) from the time of resection of the colorectal tumor. The median CEA level was 16 ng/ml (range, 1-1,292) at the time of liver resection. In 16 patients (8%) the CEA level exceeded 200 ng/ml. The median number of metastases was one (range, 1-8) with a median diameter of the largest metastases of 3 (range, 0.5-15) cm. The Clinical Risk Score was ≥3 in 60 patients

(30%). Fifty-nine patients (31%) were treated with neoadjuvant chemotherapy. AR was performed in 88 patients and a NAR was performed in 113 patients. The clinicopathological features of the AR and NAR are compared in Table 1.

Table 1: Clinicopathological variables

Variable	Anatomical	Non-anatomical	P-value
	(n=88)	(n=113)	
Age	65 (30-82)	65 (36-86)	0.585
Gender (Male)	56 (64)	70 (62)	0.806
Number of tumors	2 (1-7)	1 (1-7)	0.295
Size largest tumor (cm) [(a)]	4 (1-15)	3 (1-7)	<0.001
Bilobar distribution	20 (23)	32 (28)	0.369
CEA [(b)]	16,4 (1-1292)	15,9 (1-909)	0.078
>200 ng/ml	10 (12)	6 (5)	0.113
Time to resection			
Synchronous	35 (40)	43 (38)	0.804
Metachronous	53 (60)	70 (62)	
Disease free interval (months)	24 (4-93)	17 (4-193)	0.430
Clinical risk score [(a)]			
1-2	57 (66)	82 (73)	0.241
3-5	30 (34)	30 (27)	
Neoadjuvant chemotherapy	31 (35)	28 (25)	0.107
Site primary tumor			
Colon	55 (63)	59 (52)	0.144
Rectum	33 (37)	54 (48)	
Tumor stage primary tumor			
0-2	12 (14)	23 (20)	0.213
3-4	76 (86)	90 (80)	
Lymph node primary tumor			
Positive	45 (51)	69 (61)	0.159
Negative	43 (49)	44 (39)	

Missings: [a] = 2, [B] = 4

Data are numbers with percentages in parentheses or medians with ranges in parentheses unless otherwise indicated

Surgical treatment

A single NAR was performed in 69 of the patients (61%), while 44 (39%) had two or more NAR simultaneously. A right hemihepatectomy was the most frequently performed AR (47 resections, 43%) followed by left hemihepatectomy (15 resections, 14%). Bisegmentectomies were performed in 18 patients (21%). (Table 2)

Table 2: Type of resection

Liver resection	No. of resections	
	n= 201	(%)
Nonanatomic (*n*=113)		
Single	69	61
Two	25	22
Three	13	12
Four	4	3
Five	2	2
Anatomical (*n*=88)		
S 2-3	12	14
S 6-7	6	7
Right hemihepatecomy	47	53
Left hemihepatectomy	15	17
Extended right hemihepatectomy	4	5
Extended left hemihepatectomy	1	1
Combination of anatomical resections[a]	3	3

S segment

[a] seg 2-3 + seg 1 resection, seg 2-3 + seg 6-7 resection

Outcome

Table 3 presents the outcome of patients who underwent AR *vs*. NAR. After AR, 32 patients (36%) received a blood transfusion. This was significantly lower after a NAR (23 patients, 20%; p=0.012). The transfused patients in the AR group received a median of 3 units of erythrocytes (range, 1-6). In the NAR group the median transfusion rate was also 3 units of erythrocytes (range, 1-9), but with a larger range. The hospital stay was significantly shorter after NAR (7 (range, 1-26) days *vs*. 8 (range, 4-42) days; p <0.001). There was no significant difference in mortality rate between the two groups. Insuf-

Table 3: Outcome surgery

Variable	Anatomical	Non-anatomical	P-value
	(n=88)	(n=113)	
Blood transfusion	32 (36)	23 (20)	0.012
Hospital stay	8 (4-42)	7 (1-26)	<0.001
Complications	24 (27)	26 (23)	0.488
In-hospital mortality	2 (2)	1 (1)	0.421
Positive resection margins	8 (9)	12 (11)	0.728

Data are numbers with percentages in parentheses or medians with ranges in parentheses unless otherwise indicated

ficient capacity of the liver remnant was the cause of death in the two patients in the AR group. One patient in the NAR group died due to aspiration pneumonia. The median follow-up was 35 (range, 1-111) months in both groups. With respect to the median time to recurrence, the groups were comparable (AR group 9 (range, 1-46) months *vs*. 10 (range, 2-55) months in the NAR group; *p*= 0.802). The DFS was similar for the AR and NAR group, 56%, 38%, 30% and 60%, 39%, 32% at 1, 3, and 5 years respectively (*p*= 0.441, *p*= 0.81, *p*= 0.599) (Figure 1). The pattern of recurrence did not differ between the

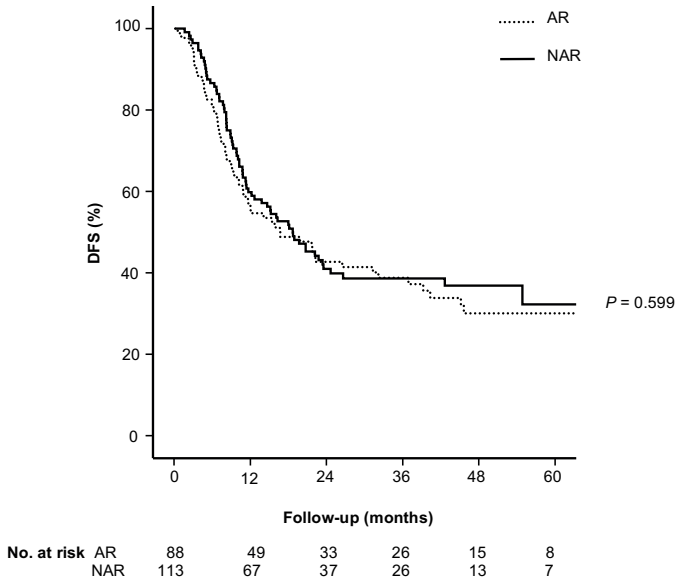

Figure 1 Disease free survival stratified by surgical procedure. Median DFS was 16.7 months in the AR group and 18.7 months in the NAR group. The 5-year DFS rate was 30% and 32%, respectively (*p*= 0.599)

two groups (Table 4). The 3-year intra hepatic recurrence rate was 37% in the AR group and 33% in the NAR group (*p*=0.620). Seventeen patients in AR group and 26 patients in the NAR group developed liver metastases limited to the liver. These patients received similar therapy (Table 4). The OS was 96%, 61%, 49% for the AR group and 97%, 65%, 39% for the NAR group at 1, 3, and 5 years, respectively (*p*= 0.715, *P*= 0.611, *p*= 0.989; Figure 2)

Table 4: Patterns of recurrence and treatment modality

	Anatomical	Non-anatomical	P-value
	(n=88)	(n=113)	
Location recurrence			
Liver	17 (30)	26 (38)	0.156
Liver + Lung	10 (18)	4 (6)	
Liver + elsewhere	2 (2)	5 (7)	
Elsewhere	28 (49)	34 (49)	
Therapy Liver Metastases			
No therapy	1 (6)	2 (8)	0.398
Systemic therapy	9 (53)	8 (32)	
Local therapy	7 (41)	15(60)	
- Resection	3	10	
- RFA	2	3	
- STBR	1	2	
-Liver perfusion	1	0	

RFA= radio frequency ablation, STBR= stereotactic body radiation

Data are numbers with percentages in parentheses or medians with ranges in parentheses unless otherwise indicated

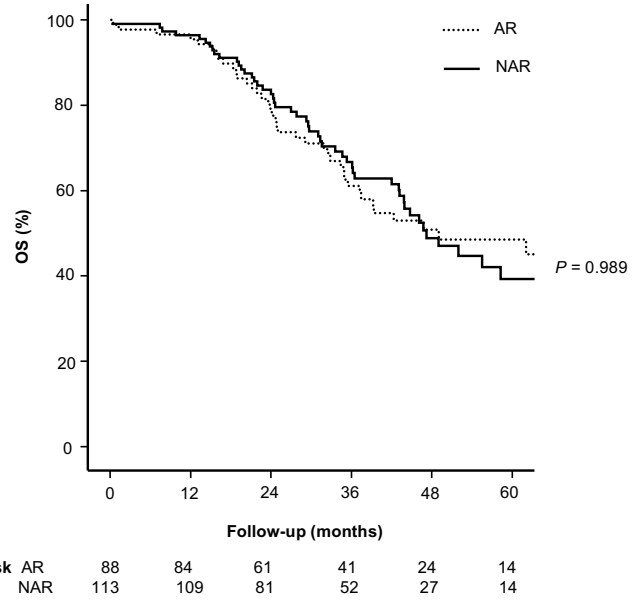

Figure 2 Overall Survival stratified by surgical procedure. Median OS was 49 months in the AR group and 47.2 months in the NAR group. The 5-year OS rate was 49% and 39%, respectively (p= 0.989)

DISCUSSION

This study demonstrates no significant difference in outcome between patients with CLM anatomical or NAR. The 5-year disease free (AR 30% *vs*. NAR 32%) and OSs (AR 49% *vs*. NAR 39%) in our study is consistent with the literature[2,24-28].

The major drawback is the retrospective nature of this study. Randomization would be difficult in this patient group, because the technique for liver resection is a tailor-made approach based on the size, number, location and distribution of the metastases. In addition, the consideration between conservation of liver parenchyma, complete surgical tumor clearance and complications is of importance in this decision. Although patients were not randomized the basic characteristics were similar as shown in Table 1.

Liver parenchymal-sparing surgery is already frequently used for CLM for several reasons. Functional hepatic reserve must be considered for any liver resection, its significance increases in the context of neoadjuvant chemotherapy, which is used to downsize the tumor load, making more patients eligible for surgery[29]. However, although chemotherapy increases resectability, it is associated with significant hepatic changes such as hepatic sinusoidal obstruction, periportal inflammation and steatohepatitis, which can affect patient outcome[15]. Specifically, chemotherapy-associated steatohepatitis is associated with the risk of progressive hepatic failure and death after resection[16]. Therefore, maximizing the amount of residual liver parenchyma is of considerable importance in patients who have had chemotherapy.Moreover, surgical stress can be reduced by non-anatomical resections, which may affect perioperative morbidity and mortality[14,25]. Several studies reported significant shorter operating times and significant less blood loss after NAR[25,26,28]. This is also seen is our study population. Patients who underwent an AR received significant more blood transfusions than the patients after a NAR. (AR 36% *vs*. NAR 20%, p= 0.012). In our series, there were three deaths within 30 days of surgery: two in the AR group and one in the NAR, which was not significantly different. There are studies suggesting more postoperative deaths in the AR group[2,25,26,28]. It is important to note that postoperative mortality is a rare event and that these studies are not powered to compare this.

The possibility to treat recurrent CLM with local therapy, such as repeated hepatectomy[17], RFA[11] or STBR[30] is a great benefit of the parenchymal sparing method. In our study, disease recurrence in the liver was similar for both AR and NAR (51%). The re-intervention rate for CLM was higher in the NAR group (AR 41% *vs*. NAR 60%) Although this number does not reach significance, probably due to the small numbers, our findings suggest that local treatment for intra-hepatic recurrences is more often possible in the parenchymal sparing method. Our findings are consisted with the literature which states that re-interventions for CLM increases the survival after disease recurrence[31-33]. For this reason close surveillance of patients after NAR is essential. One of the possible disadvantages

of NAR reported in the literature by DeMatteo *et al*. is the higher incidence of positive resection margins[24]. In more recently published literature, it is advocated that a resection margins <1 cm is no longer a contra-indication for a curative resection. Moreover, recent literature suggests that size of surgical margin does not correlate significantly with DFS or OS; even the need for R0 resections is being discussed[34,35]. In a study by de Haas *et al.* the 5 year overall survival was similar for patients after a R0 or a R1 resection (61% *vs*. 57%; $p = 0.27$) although the recurrence was higher in the R1 group (28% *vs*. 17%; $p = 0.004$)[6]. In our study the R1 resection rate was 9% in the AR group and 11% in the NAR group, which is comparable to the literature[6,27]. The concept of performing limited NAR with narrow margins is supported by the fact that micro metastases in the liver parenchyma surrounding CLM are rare and are primarily confined to the immediate surrounding area of the tumor border[36,37].

The second possible drawback of NAR which is postulated in the literature[24] is the lack of vascular control. This is the opposite of what is published in the past years. Blood loss and blood transfusions are reported to be significant less during and after NAR, which is also confirmed by our results[25,26,28].

In contrast to CLM, some studies report AR to be superior NAR in HCC[18-20]. This difference may be explained by the variation in disease biology seen in primary versus metastatic liver tumors. Metastatic liver lesions develop from blood-borne tumor cells circulating throughout the body. AR may not offer the same advantage for these lesions as for HCC, which arise within a segment of the liver and might benefit from the removal of the complete functional liver unit.

Multiple studies have been conducted to investigate which resection is favorable for patients with CLM: anatomical or a nonanatomical. Most authors similarly conclude that there is no significant difference between AR and NAR in DFSs an OSs. A disadvantage of the majority of studies is that the patient characteristics are not comparable between the two groups regarding tumor size and number, nodal status of the primary tumor, disease free interval and CEA blood levels[2,14,25,26]. Our study contributes to this discussion due to the use of the Clinical Risk Score (CRS) in which the previous described characteristics are incorporated. The CRS is the same for the AR and NAR which indicates that the groups are comparable.

Furthermore the use of different neoadjuvant chemotherapy regimens over the years makes it difficult to compare the results of the studies[2,14,26-28]. We started our patient selection after 2000, because Irinotecan and Oxaliplatin were added to the chemotherapeutic arsenal from this year and all patients were treated with effective chemotherapeutics.

We conclude that with a comparable complication rate, less blood transfusions, a significantly shorter hospital and comparable DFS and OS rates, a NAR is a save technique for the resection of CLM.

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Chapter 7

Circulating tumor cells and sample size: "The more, the better"

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Adapted from:
Circulating tumor cells and sample size: "The more, the better"
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INTRODUCTION

Jiao *et al*. studied the presence of circulating tumor cells (CTCs) in blood of patients with colorectal liver metastases (CRLM) by automated immunomagnetic enrichment and image cytometry using the CellSearch system (Veridex, Raritan,NJ)[1]. They showed CTCs to be present in the hepatic macrocirculation in significantly higher numbers than in the peripheral circulation (median 187, (range, 0-500) *vs*. median 1, (range, 0-6)). Despite the number of evaluated patients in this study being small, the low number of detected CTCs in the peripheral circulation suggest that CTC enumeration and characterization plays no role in this specific patient population.

Enumeration and in particular characterization of CTC holds great promise for patient management and research purposes[2]. Of several assays enabling CTC detection (reviewed by Mosterd *et al.*[3]), the CellSearch system has been approved by the US Food and Drug Administration for use in metastatic breast, prostate and colorectal cancer. According to the manufacturer's instructions, CTC enumeration should be performed in 7.5 ml blood. In the first study of 196 patients with metastatic colorectal cancer whose CTCs were measured with the CellSearch system. At least 2 CTCs per 7.5ml blood were detected in 30% of patients, whereas only 17% had ≥ 5 CTCs per 7.5 ml blood[4]. In a subsequent publication by Cohen *et al*. investigating the prognostic role of CTCs in advanced colorectal cancer, patients with a CTC count above a threshold of 3 CTCs per 7.5ml blood had a worse outcome after systemic therapy compared with patients with lower CTC counts. The 430 patients in this study received first-, second- or third-line chemotherapy, of whom 26% had a CTC count ≥ 3 CTC threshold, whereas 48% of the patients had ≥ 1 CTC per 7.5ml blood[5]. In a third study in 451 metastatic colorectal cancer patients, at least 3 CTCs per 7.5 ml blood were detected in 29% of the patients[6]. From these studies we can conclude that the number of detectable CTCs in patients with advanced colorectal cancer using the CellSearch System is low to even below detection limit, which is in contrast to other tumor types. In metastatic breast and prostate cancer, the percentage of patients with a CTC count of ≥5 CTCs per 7.5ml was 66% and 49%, respectively[7,8]. Not surprisingly and in line with the findings of Jiao *et al.*, in nonmetastastic colorectal cancer, the number of patients with detectable CTCs is even lower than in advanced disease[1]. One study revealed ≥ 2 CTCs/7.5 ml in two of 11 patients[9], whereas in another study CTCs could be identified in only two of 31 patients[10].

The inability to detect CTCs in more patients with cancer with the currently available methods can have several causes. CTC detection could be limited by technical difficulties, such as the absence of marker expression required for CTC detection[11], but could also be a true reflection of the CTC frequency. The low number of detected CTCs in patients with colorectal liver metastases (CLM) compared with overt metastatic disease prompted us

to explore whether analyzing a larger blood volume would improve CellSearch-based CTC detection in patients with CLM.

PATIENTS AND METHODS

In 15 patients undergoing liver resection and/or open radio frequency ablation, we obtained, before operation, 40 ml blood from a peripheral arterial line. Blood was collected in 10 ml evacuated tubes (CellSave tubes; Veridex) and pooled, stored at room temperature and processed within 72 hours of collection according to the standard operating procedure[4].

For each patient we compared the number of detected CTCs in 7,5 ml blood to the number found in 30 ml blood. We performed a modified Ficoll density gradient separation to reduce the 30 ml blood to a volume of 7,5 ml enriched blood[12].

In short, samples were processed on the semi-automated CellTracks AutoPrep System using the CellSearch Epithelial Cell Kit (both Veridex LLC). To immunomagnetically enrich epithelial cells from whole blood, magnetic beads coated with anti-epithelial-cell adhesion molecule were used. The remaining cells are then stained with the nuclear dye 4′,6-diamidino-2-phenylindole (DAPI), anti-cytokeratin (CK) 8/18/19 antibodies labelled with phyco-erythrin (PE), and anti-CD45 antibodies labelled with allophycocyanin (APC). The samples are transferred to a Magnest Cell Presentation Device (Veridex LLC), where the cells are scanned by the CellSpotter Analyzer (a four-color semi-automated fluorescence microscope). Cell images were evaluated by skilled readers and all cells fulfilling all criteria for a CTC - size of ≥4μm, round to oval morphology, positive staining for CK-8/18/19, a visible nucleus (DAPI positive), at least 50% overlap between nucleus and cytoplasm, and negative staining for CD45- were selected.

The statistical analyses were carried out using the statistical software package SPSS (version 15, SPSS Inc., Chicago USA). Continuous variables are presented as median (range). Categorical variables are presented as number (percentage). For comparison of continuous data, the independent sample *t*-test was used. In all analyses the significance level was set at $p<0.05$.

RESULTS

In 7.5 ml blood, the median number of CTCs was 1 (range, 0-4). In 30 ml blood, the median number of CTCs was significantly higher (median 2 CTCs; range, 0-9; $p = 0.03$). The median paired CTC difference between 7,5 ml and 30 ml blood was 1 (range, -1 to 7). The median CTCs and range of the 7,5 ml, 30 ml samples and the paired difference are

shown in figure 1. Analyzing 7,5 ml blood ≥1 CTC was found in 10 patients (67%) and ≥3 CTCs were found in two patients (13%). In 30 ml blood, ≥1 and ≥3 CTCs were detectable in 13 (87%) and seven patients (47%), respectively (figure 2).

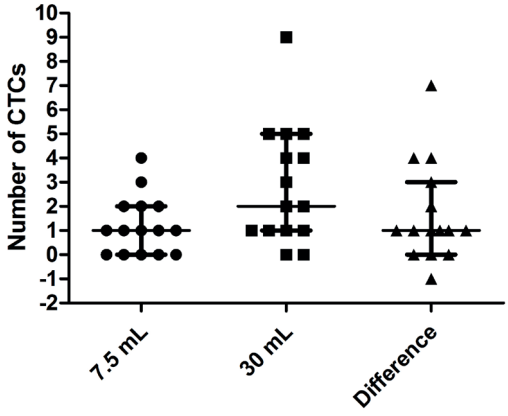

Figure 1 The distribution of the CTC counts is shown of the 7,5 ml, 30 ml blood samples and paired difference. The horizontal lines across the samples depict the median and interquartile range.

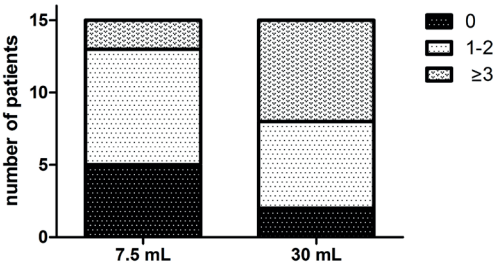

Figure 2 Depicted are the categorical data of the CTC enumeration in the 7,5 ml and 30 ml blood samples.

DISCUSSION

In this study we demonstrate that significantly more CTC's can be recovered in 30ml blood compared to 7,5ml blood using an enrichment step, prior to enumeration with the CellSearch System in patients with CLM.

The CellSearch system samples 7.5ml volume of blood, which represents ~0.15% of the total blood volume in an average patient. The sensitivity of the Cell Search system is thus limited by both statistical considerations and the blood volume that can be tested.

Knowing that CTC's have been observed in the peripheral blood of cancer patients at very low concentrations of 10^{-7}- 10^{-8} of normal peripheral blood cells[13], enlarging blood volume and adding an enrichment step in the sample preparation is only a logical tactic.

Ficoll density gradient separation is a commonly used method and reported on since the sixties[14]. This technique is easy applicable, can be used for all tumor types and is inexpensive. One of the major advantages is that cells are undamaged after Ficoll density gradient separation and the nucleus is intact[15]. Especially in combination with the CellSearch system this is of great importance; the images captured by the CellSpotter Analyzer, are judged for CTC criteria, including size (≥4μm), morphology (round to oval) and visible nucleus presence.

In our study we did not perform repeated measurement as this Ficoll enrichment technique in combination with the Cell Search was previously tested and presented by Gross et al.[12] .

The number of CTCs in CRC is relatively low, particularly when compared to breast and prostate cancer[1,4,5,9,10]. This difference can be explained by tumor biology. Breast cancer has been well described as a systemic disease from diagnosis, even in patients with localized tumors, micrometastases can be visualized in the bone marrow using immunohistochemistry techniques[16]. Prostate cancer has a similar biology, 25% of men with clinically localized disease have micometastases in bone marrow[17]. Both of these diseases are often accompanied by hematogenous metastases to the bone. In CRC the most frequent site of metastases is the liver via portal vein drainage. This concept is supported by data of Jiao *et al.*[1], who find higher numbers of CTCs in the portosystemic circulation than in peripheral blood. The low number of CTCs is also seen in other gastrointestinal malignancies, such as gastric and esophageal cancer, probably caused by filtration of these cells by the portal circulation[9].

Another explanation for the small amount of retrieved CTCs in CRC could be the dependence of the Cellsearch System on antibodies to EpCAM. EpCAM, short for epithelial cell adhesion molecule, is expressed on the surface of epithelial cells. It has been reported that tumor cells that have gained entry to the bloodstream can undergo epithelial-mesenchymal transition (EMT). In this process tumor cells lose their epithelial traits including cell-cell adhesion, apical-basal polarity, and lack of motility and acquire mesenchymal properties such as motility, invasiveness, and a resistance to apoptosis[18]. With the loss of cell-cell adhesion, epithelial cell adhesion molecules such as EpCAM are down regulated[19]. In a study by Rao et al. the EpCAM expression was 10-fold less in CTCs compared to primary and metastatic tissues[20]. By using EpCAM antibodies, the CellSearch System is unable to detect the EMT positive CTCs and can give an underestimation of CTCs.

Though this study is based on a relatively small number of patients, it shows that more CTCs can be detected in CRLM patients when using 30 ml instead of 7.5 ml pe-

ripheral blood. This more frequent CTC detection renders it worthwhile to explore CTC enumeration as a prognostic factor in this patient population. In addition, detection of CTCs in the majority of patients with CLRM may allow for more widespread molecular CTC characterization. Characterization is already possible using a single CTC and might contribute to additional individualization of patient management[21]. We believe that future clinical studies in CRLM patients should be performed using 30 ml blood, thereby making the potential benefits of CTC detection and characterization available for more patients.

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Chapter 8

Prognostic value of circulating tumor cells for early recurrence after resection of colorectal liver metastases

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ABSTRACT

Background: Despite good outcomes for many, a substantial group of patients undergoing metastasectomy for isolated liver metastases from colorectal cancer (CRC) experience early recurrence. We have investigated whether circulating tumor cell (CTC) detection can identify patients developing disease recurrence within 1 year after liver metastasectomy.

Methods: In CRC patients undergoing liver metastasectomy, 30ml peripheral blood was withdrawn preoperatively. CTCs were detected by the CellSearch system after a density gradient-based enrichment step.

Results: 173 samples from 151 individual patients were analyzed. In 75 samples (43%), CTCs were detected, 16% had ≥3 CTCs/7.5ml of blood. Eighty-two patients (47%) experienced early disease recurrence (<1 year). The 1-year recurrence rate between patients with or without detectable CTCs were similar (47% *vs*. 48%) or with a low or high CTC count (<3 or ≥3 CTCs/7.5 ml of blood) (50% *vs*. 47%). Also disease-free and overall survival were similar between patients with or without CTCs.

Conclusion: The presence of CTCs in preoperative peripheral blood samples does not identify patients at risk for early disease recurrence after curative resection of colorectal liver metastases. Other parameters are needed to better identify patients at high risk to relapse after liver metastasectomy for CRC.

INTRODUCTION

In Western countries, colorectal cancer (CRC) represents one of the most common malignant diseases and forms a substantial cause of death, frequently due to liver metastases. For patients presenting with isolated colorectal liver metastases (CLM) and disease amenable for complete resection, liver metastasectomy is a potentially curative approach yielding 5-year survival rates, of up to 60% in highly selected patients[1,2]. The indications for liver resection have expanded over the past decade due to improvements in surgical techniques, the introduction of more effective systemic chemotherapy and the use of portal vein embolization, radio frequency ablation and stereotactic radiation. And in carefully selected patients, extra-hepatic disease is even no longer a contra-indication for local therapies.[3-9].

However, despite the good outcomes for many patients undergoing this procedure, there is still a substantial group of patients encountering early recurrence. In a retrospective analysis performed in our center, the 1-year recurrence rate of CLM after liver resection was almost 50%[10]. Obviously, the identification of patients at high risk of disease recurrence after surgery for resectable CLM might lead to better selection of patients for this procedure. These patients should either be spared a potentially futile surgery or, their condition permitting, additional and more intensified therapy should be explored to minimize the risk of early relapse.

Circulating tumor cells (CTCs) are cells present in the peripheral circulation of the majority of metastatic cancer patients. Several techniques are currently available for their measurement, but of these, only the CellSearch technique (Veridex LLC, Raritan, NJ) has been approved for use as a prognostic marker in metastatic breast, prostate and CRC by the US Food and Drug Administration. Using this method, the presence of CTCs in peripheral blood of patients has strong prognostic value in various malignancies including metastatic CRC[11-15]. Most studies on the prognostic value of CTCs in metastatic CRC include patients with irresectable disease treated with chemotherapy[14,16,17]. However, concerning patients with isolated CLM undergoing liver metastasectomy, little is known about the possible prognostic value for CTCs.

The objective of this study was to evaluate whether the detection of CTCs by the CellSearch System can identify patients with resectable CLM undergoing liver metastasectomy who will develop disease recurrence within 1 year after surgery.

METHODS

Patients

Patients over 18 years of age with an adenocarcinoma of the colon or rectum with metastases confined to the liver confirmed by computed tomography (CT) scan or magnetic resonance imaging (MRI)) and eligible for liver resection or "open" radio frequency ablation therapy (RFA) were included. Patients were excluded if complete resection of the liver metastases was not possible in one procedure, the primary tumor was *in situ* (liver-first approach or synchronous resection of primary tumor and liver metastasis), extrahepatic metastasis, histological examination of the liver specimen showed no CLM, or follow-up after resection was <1 year. Ours is a referral hospital; preoperative chemotherapy is not administered as a standard treatment protocol for patients with resectable CLM according to the Dutch Guidelines. Most of our patients have already received neoadjuvant chemotherapy in the referring hospital. In our center, the indication for neoadjuvant chemotherapy is two-fold: in case of initially difficult/unresectable liver metastases, or in case of multiple (>4) synchronous metastases. None of the included patients received adjuvant chemotherapy after the curative liver resection. Clinical data were collected from the medical records and included demographic details, maximum size, number and distribution of liver metastases on CT scan or MRI, plasma carcinoembryonic antigen (CEA) levels, pre-operative chemotherapy (chemotherapy administration within 6 months prior to resection), Fong Clinical Risk Score (CRS)[18], location and pathological TNM stage of the primary tumor.

This study was approved by the Ethical Board of the Erasmus MC (METC 2006-089) and all patients gave their written informed consent.

Blood sample analyses

Thirty ml of blood was drawn from the peripheral arterial line directly preoperatively, before manipulation of the tumor, by the anaesthesiologist in "CellSave" tubes (Veridex LLC). Samples were stored at room temperature and analyzed within 96 hours after collection. The three blood tubes were pooled and then reduced to a volume of 7.5ml by Ficoll density-gradient separation as previously described[19,20]. CTCs were enumerated using the CellSearch System according to the manufacturer's instructions[21]. Briefly, samples were processed on the semi-automated CellTracks AutoPrep System using the CellSearch Epithelial Cell Kit (both Veridex LLC), which contains magnetic beads coated with anti-epithelial-cell adhesion molecule (EpCAM) antibodies to immunomagnetically enrich epithelial cells from whole blood. Remaining cells are then stained with the nuclear dye 4',6-diamidino-2-phenylindole (DAPI), anti-cytokeratin (CK) 8/18/19 antibodies labelled with phyco-erythrin (PE), and anti-CD45 antibodies labelled with allophycocyanin. After transferral of the sample to a Magnest Cell Presentation Device (Veridex

LLC), the cells are scanned by the CellSpotter Analyzer, a four-color semi-automated fluorescence microscope. Presented images were assessed by trained readers and all cells fulfilling all criteria for a CTC - size of ≥4µm, round to oval morphology, positive staining for CK-8/18/19, a visible nucleus (DAPI positive), at least 50% overlap between nucleus and cytoplasm, and negative staining for CD45- were selected.

Follow-up

After hepatic resection, patients routinely underwent a physical examination and determination of CEA-level, abdominal/chest CT or ultrasonography every 4 months for the first year, every 6 months the second year and once a year thereafter.

Statistical analysis

The primary endpoint of this study is disease recurrence within 1 year after hepatic resection for CLM. In a pilot study, 50% of a patient group with characteristics similar to the population studied here tested positive for the presence of CTCs[20]. To detect a 20% difference in 1-year recurrence rate between patients with detectable CTCs *vs* those without detectable CTCs with a power of 80% and a significance level of 5%, knowing that the overall 1-year recurrence rate is approximately 50%, 200 patients had to be included.

We use the presence of CTCs (≥1 CTC) as well as high CTC count (≥3 CTCs) as a cut-off points for analyses in line with Allard *et al.* and Gazzaniga *et al*.[21,22]. Categorical data were described as counts with percentages between brackets and continuous data as median with the range between brackets. The χ^2-test was used to compare categorical data, for continuous date the independent sample *t*-test and if data were not equally distributed the Mann-Withney *U*-test was used. Disease free survival (DFS) was defined as the interval elapsing between the day of surgery to the day of recurrence, death or censoring at most recent follow-up. Overall survival (OS) was the time between the day of surgery and the day of death or censoring at most recent follow-up. Survival analyses (DFS and OS) were executed following the Kaplan-Meier method and comparisons were made using the log rank test. A linear regression model was used to evaluate the association between CTC count and the time interval from the last dose of chemotherapy until the operation. In all analyses the significance level was set at $P<0.05$. All the statistical analyses were two-sided and carried out using the statistical software package SPSS (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY, USA)

RESULTS

Patients

Blood was drawn from all patients (included in the METC 2006-089 trail) undergoing a liver metastasectomy for isolated CLM, between 1 June 2008 and 31 May 2012. Out of the in total 343 blood samples from 289 individual patients collected, 170 samples were excluded (Figure 1). After applying these exclusion criteria, 173 blood sample of 151 individual patients were left for evaluation. Twenty-two patients underwent a second liver resection for recurrent disease confined to the liver following the first resection. Also these samples were included and were considered as separate cases. Patients were divided into two groups according to disease recurrence <1 year (*n*=82) and disease recurrence ≥1 year or no recurrence (*n*=91) following hepatic surgery (Table 1). There is a significant difference between the groups in the number of metastases (2 (range, 1-10) *vs*. 1 (range, 1-8) *p*=0.04), as well as the ratio between synchronous, metachronous and recurrent liver metastases, with higher percentages of metachronous metastases in the group with late or no disease recurrence (42% *vs*. 60%, *p*=0.02). Also the distribution of the Fong CRS showed significant difference, which was, as expected, higher in the early recurrence group (40% score 3-5 *vs*. 21%, *p*=0.01).

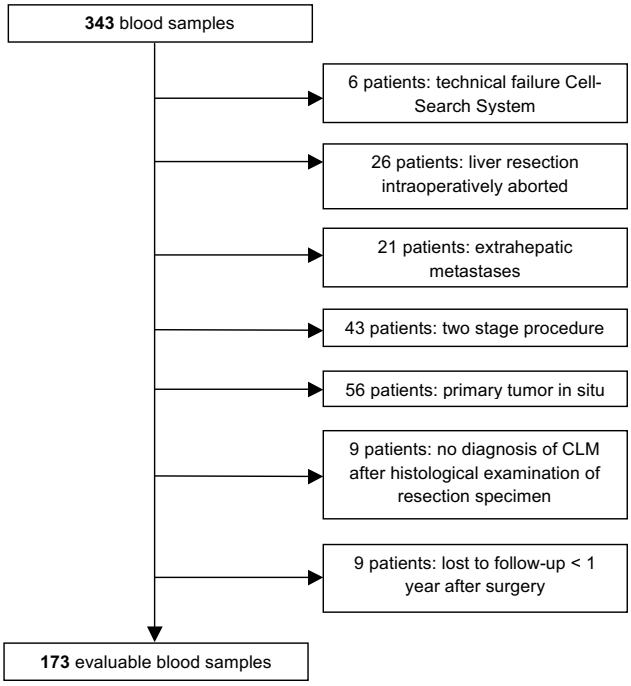

Figure 1 Flow chart sample inclusion

Table 1: Basic characteristics of the study population divided by recurrence <1 year versus no recurrence or later than 1 year

	Total inclusion n=173		
Variable	**Recurrence < 1 year (n=82)**	**No recurrence or recurrence > 1 year (n=91)**	***P*-value**
Age (years, median (range))	63 (36-81)	65 (37-84)	0.26
Gender (male)	52 (63%)	59 (65%)	0.85
Liver metastases			
Number of tumors (median (range) [§]	2 (1-10)	1 (1-8)	**0.04**
Size largest tumor (cm, median (range))[§]	2.8 (0.2-18)	2.6 (0.2-10)	0.24
Bilobar distribution	27 (33%)	28 (31%)	0.76
Time to resection			
synchronous	27 (33%)	25 (28%)	**0.02**
metachronous	34 (42%)	55 (60%)	
recurrence	21 (25%)	11 (12%)	
Clinical risk score [§]			
0-2	46 (60%)	69 (79%)	**<0.01**
3-5	31 (40%)	18 (21%)	
Preoperative chemotherapy	41 (50%)	39 (43%)	0.35
Primary tumor			
Site primary tumor [§]			
colon	59 (72%)	66 (73%)	0.84
rectum	23 (28%)	24 (27%)	
Tumor stage primary tumor[§]			
0-2	11 (14%)	12 (13%)	0.94
3-4	69 (86%)	78 (87%)	
Lymph node primary tumor[§]			
Positive	49 (60%)	45 (50%)	0.17
Negative	32 (40%)	45 (50%)	

§ missing data

CTC enumeration and primary endpoint

In Table 2 the results of the CTC analyses are shown. CTCs were detected in 43% of the blood samples and the median number of CTCs was zero (range, 0-49) in all samples. With respect to the primary objective, there was no significant difference in the 1-year recurrence rate between patients with (≥1 CTC) or without CTCs (47% *vs*.48%; *p*=0.87). The 1-year recurrence rate in the group with ≥3 CTCs also showed no significant difference compared to the <3 CTC group (50% *vs*. 47%; *p*=0.76). We performed the analyses on a per patient basis as well (151 samples); this showed similar results (data not shown).

Table 2: Results CTC enumeration divided by recurrence <1 year versus no recurrence or later than 1 year

	Total inclusion n=173		
Variable	**Recurrence < 1 year (n=82)**	**No recurrence or recurrence > 1 year (n=91)**	***P*-value**
CTCs			
Number (median, range)	0 (0-28)	0 (0-49)	0.70
CTCs present	35 (43%)	40 (44%)	0.87
CTCs ≥3	14 (17%)	14 (15%)	0.76

CTC = circulating tumor cell

Survival analyses

The median follow-up for all 151 patients was 28 months (range, 12-59 months), and the median DFS, calculated for every blood sample (*n*=173) was 14 months (95% CI 10.9-17.2 months). Disease recurred in 115 cases (66%), of which 82 (47%) were within 1 year. There was no significant difference in DFS between patients with no or ≥1 CTCs (*p*=0.56) or between those with <3 or ≥3 CTCs (*p*=0.34, Figure 2a and 2b). During the follow-up period 40 patients (26%) died. Survival analyses showed no significant difference in OS between patients with no or ≥1 CTCs (*P*=0.96) or between those with <3 or ≥3 CTCs, (*p*=0.17, Figure 3a and 3b).

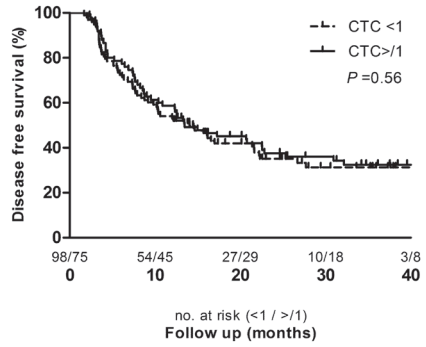

Figure 2a Disease-free survival (DFS) stratified by CTC presence (≥1 CTC). Median DFS was 13.5 months (95% CI 5,9-21,1) when CTCs were present and 14 months (95% CI 8,8-19,4) when CTCs were not present. There is no significant difference when the groups are compared (*P*=0.56).

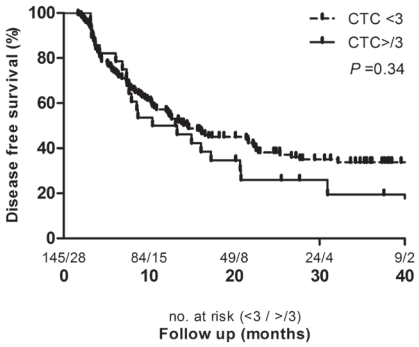

Figure 2b Disease-free survival (DFS) stratified by high CTC count (≥3 CTC). Median DFS was 10.4 months (95% CI 2,5-18,4) when ≥3 CTCs were present and 14.5 months (95% CI 7,8-21,3) when there were less than 3 CTCs. There is no significant difference when the groups are compared (*P*=0.34).

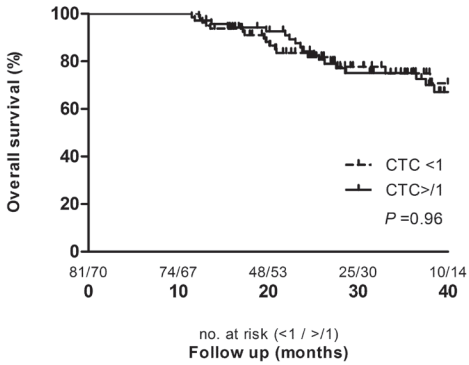

Figure 3a Overall survival (OS) stratified by CTC presence (≥1 CTC). There is no significant difference in CTC presence in relation to overall survival (*P*=0.96)

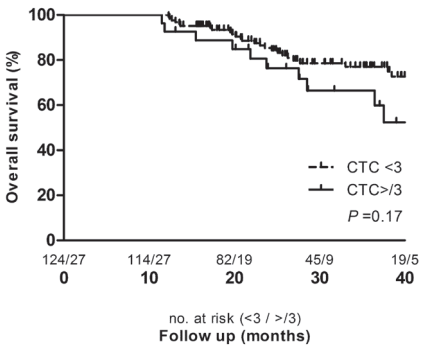

Figure 3b Overall survival stratified by high CTC count (≥3 CTC). There is no significant difference in high CTC count in relation to overall survival (*P*=0.17)

Subgroup analyses

A subgroup analysis was performed for the CTC count between patients who did (*n*=80) and did not (*n*=93) receive preoperative chemotherapy. Patients in the preoperative che-

motherapy group, received a median number of four cycles chemotherapy (range, 2-12). The median time interval between the last dose of chemotherapy and the operation was 8.6 weeks (range, 2.7-38 weeks). The results concerning the CTC count are shown in Table 3. The median CTC count was lower in patients who received chemotherapy (p=0.05) (Figure 4). In 36% of the patients who received preoperative chemotherapy CTCs were detected (≥1CTC), compared to 50% of patients who did not receive chemotherapy, this difference was not statistically significant (p=0.08). The percentage of patients with a high (≥3) or low (<3) CTC count also showed no difference between the groups (p=0.22). The 1-year recurrence rate for patient who received preoperative chemotherapy was not significantly different between patients with or without CTCs present (48% *vs*. 53%, p=0.69). There was no association between CTC count and the time interval from the last dose of chemotherapy until the operation (for each week increase in time interval between the last dose of chemotherapy and the operation, the CTC count increases by 0.067 CTCs (95% CI: -0.008, 0.142; p= 0.08). For patients who did not receive preoperative chemotherapy the 1-year recurrence was similar between the group with and without CTCs (46% *vs*. 43%, p=0.76)

Table 3: Results CTC enumeration divided by preoperative chemotherapy

	Total inclusion n=173		
Variable	**With preoperative chemotherapy (n=80)**	**Without preoperative chemotherapy (n=93)**	***P*-value**
CTCs			
Number (median (range))	0 (0-13)	1 (0-49)	**0.05**
CTCs present	29 (36%)	46 (50%)	0.08
CTCs ≥3	10 (13%)	18 (19%)	0.22

CTC = circulating tumor cell

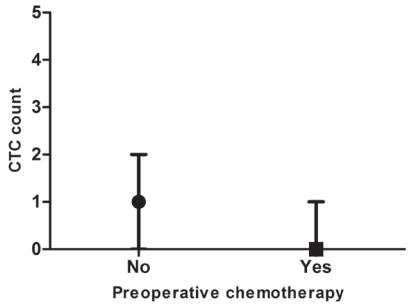

Figure 4 CTC counts in patients who received preoperative chemotherapy (n=93) and patients who did not (n=80), depicted are the medians with the interquartile range.

DISCUSSION

This study does not demonstrate a prognostic value for CTC enumeration in patients with isolated CLM undergoing a partial liver metastasectomy with a curative intent. The power analysis conducted prior to our study indicated that 200 patients had to be included to show a 20% difference in the relapse rate at 1 year between patients with *vs* those without CTCs. Of the 343 patient samples, 170 cases had to be excluded for several reasons including extra-hepatic disease or residual disease after surgery. We excluded these patients as we expected their remnant tumor tissue to influence the course of disease. Nevertheless, despite this slightly reduced power, the DFS curves clearly show no differences in outcome between patients with *vs* those without detectable CTCs. We therefore feel that further studies using the same technique and similar study design and setting are not justified as futility has been adequately demonstrated.

The lack of prognostic value of CTC counts in this patient group with limited metastatic disease is in contrast to findings in patients with advanced CRC. In these studies where the same CTC enumeration technique was used, a CTC count of ≥3 per 7.5ml of blood was associated with a worse progression-free and OS among patients with advanced CRC treated with first-, second- or third-line chemotherapy[14,23]. Importantly, instead of drawing and analyzing 7.5ml of blood as was done in these previous studies, we drew 30ml of blood and then reduced this to a volume of 7.5ml of enriched blood by density-gradient separation. We choose this approach as we expected CTC counts to be lower in our patient group with limited metastatic tumor load. The potential benefit of this method was shown in a pilot study among 15 patients, in which more CTCs were detected using 30ml instead of 7.5ml blood (median paired difference 1 (range, −1 to 7))[20]. As a consequence, when we compare our CTC counts to other studies detecting colorectal CTCs using CellSearch in 7,5ml of blood, the difference in blood volume should be taken into account. Despite the higher blood volumes in our study, both the number of patients with CTCs present (≥1 CTC) and the number with a high CTC count (≥3) is clearly lower in our study population compared to other studies in patients with metastatic CRC. In our study CTCs were present in 43% of the patients and 16% had ≥3 CTCs, in the study of Tol *et al.* 29% of the patients had ≥3CTCs, Cohen *et al*. reported 31% ≥3CTCs and Hiraiwa *et al*. 41.4% ≥2 CTCs[14,16,24]. This difference is likely due to the fact that our study patients have relatively limited metastatic disease, as they had to be eligible for liver surgery with curative intent. This is in stark contrast with the aforementioned other studies which included patients treated up to third-line chemotherapy and who are therefore likely to have more extended disease. It has been demonstrated that more advanced disease is associated with larger amounts of CTCs[24]. Hiraiwa *et al*. found a significantly higher percentage of patients with ≥2 CTCs among patients with metastases confined to the peritoneum compared to patients with metastases to the liver and lung

(64 % *vs*. 26%, $p<0.01$). Similar results have recently been published by Kaifi *et al*.; in their case series 60% of patients with diffuse metastases had CTCs present, in contrast to patients with metastases confined to the lung and the liver (11% and 32% respectively ($p< 0.01$))[25].

Numerous articles report on the impact of chemotherapy on CTCs, and in the vast majority of patients undergoing chemotherapy, CTC numbers decline during treatment[14,26]. To exclude the possibility of the impact of neo-adjuvant chemotherapy obscuring the prognostic value of CTCs in this setting, associations were explored in only the patients receiving neo-adjuvant chemotherapy as well as those who were untreated before liver metastasectomy. But also in these separate groups, CTC counts had no prognostic value.

Only recently Seeberg *et al*. published an article were also the CellSearch was used as a method to detect CTCs (in 7.5ml blood) in patients with isolated CLM. The recurrence rate was similar to our study group. They reported that CTCs can predict nonresectability and impaired survival. CTC positivity was significantly higher in nonresectable (46%) than in resectable patients (11.7%), $p<0.01$. Contrary to our findings, patients who underwent resection and with two or more CTCs experienced reduced time to relapse or disease progression ($p<0.01$). As we used 30ml blood, had a different, predefined primary end point and used other cutoff values the studies are not fully comparable. A major difference between the studies is that they also included patients who were not eligible for resection. It is also not clear if the patients who underwent liver surgery had extrahepatic disease. Therefore, our group is more homogeneous[27].

Three studies have looked in this population (resectable CLM) using other CTCs enumeration tests based on RT-PCR assays. Vlems *et al*. and Topal *et al*. could not predict disease recurrence in patients with resectable CLM using their CTC detection methods[28,29]. A third study by Koch *et al*. did show that intra-operatively detected CTCs were of prognostic value[30]. It should be noted that these studies concerned only small numbers of patients and that the prognostic value found in latter study is yet to be confirmed. In general, the nature of RT-PCR based CTC detection assays confers to a higher sensitivity compared to the CellSearch technique, due to the fact that the CellSearch System uses very stringent criteria by which a CTC is defined. In this way, small tumor fragments, apoptotic CTCs or CTCs with low expression of one of the detection markers are not counted as CTCs. This idea is supported by our previous findings in which tumor DNA and RNA could be detected using RT-PCR, whereas using the CellSearch technique in the same blood samples, no CTCs could be detected[31]. Especially in patients with low tumor load, more sensitive CTC detection methods such as RT-PCR based methods should be explored for their value as prognostic marker, while remaining aware of concerns about test specificity. In addition to RT-PCR based methods, various other techniques to enumerate CTCs have recently been described. In contrast to the CellSearch technique, which amongst others relies on EpCAM and CK 8/18/19 expression on CTCs to be identi-

fied, other methods use size-based enrichment methods and/or other antibodies to detect CTCs. This may lead to a higher sensitivity to detect CTCs in CRC patients. Also these techniques should be explored for their value in this patient setting.

In our study we have used 30ml blood which was reduced to 7.5ml for the CellSearch analyses. With the idea that CTC detection is more frequent when sample volume is larger. This is shown when we compare the data on CTC presence between Seeberg *et al.* and ours (19,6% *vs.* 43%). Coumans *et al.* investigated different methods to increase the detection of CTCs and undescribed our method. They state that by statistical analysis of the distribution in 7.5ml of blood detected by CellSearch in patients with metastatic cancer the sample size should be 5 l of blood for the detection of CTCs[32]. Knowing that there are organ systems which have the ability to filter CTCs it is also the question if peripheral blood sampling alone is sufficient[33].

In conclusion, in this relatively large study no prognostic value for CTC counts as determined by the CellSearch technique could be found for outcome following liver metastasectomy in patients with resectable isolated CLM. The relatively high relapse rate in this group of patients with more than 50% of the patients relapsing within 1 year again underlines the high need for novel biomarkers to guide treatment in patient category.

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Chapter 9

Summary of the thesis

Nederlandse samenvatting

Summary of the thesis

Colorectal cancer (CRC) is one of the most common forms of cancer with annually, 1.36 million new patients and 694 000 deaths[1]. The liver is the most common organ affected by metastatic disease. Disease staging is performed by the TNM classification system and therapeutic strategies are based on this system[2]. Following international guidelines, patients with stage II CRC are not offered adjuvant chemotherapy after surgical resection of the primary tumor, while 25-30% of the patients will develop distant metastases[3]. For stage III CRC patients adjuvant chemotherapy is part of the standard care. Unfortunately, only a small portion (5-15%) of these patients will benefit from this therapy which is accompanied by toxic side effects[4]. The existing tools for identification of individual patients with high risk for disease recurrence are not sufficient, therefore regular follow-up visits plus blood analyses and liver imaging are necessary[5,6]. Although this follow-up regimen is expensive and time consuming it may be of value if curative treatment can be offered when liver metastases are discovered in an early stage.

Due to improvements in surgical techniques, additional treatment modalities and more effective systemic chemotherapy more patients are eligible for curative liver resection[7-13]. Unfortunately, the 1-year recurrence rate after liver resection is up to 50%[14]. When these patients could be identified at forehand, they could be spared an operation or treated with systemic chemotherapy to prevent extension of metastatic disease.

This thesis discusses clinical and biomarker approaches to determine the outcome of patients with colorectal cancer and patients with colorectal liver metastases.

Currently, information on the course of CRC disease depends on the TNM classification, subtracted from the pathology report[2]. In addition, 5 parameters are reported, including pT-stage, tumor differentiation grade, lymphovascular invasion (LVI), tumor perforation and lymph node metastasis status. These characteristics are being used to identify high risk stage II patients. In this high risk population adjuvant chemotherapy may be considered[3]. However, it's pivotal to adequately report on these parameters if subsequent treatment may depend on the outcome of the staging. **In chapter 2**, 356 pathology reports of stage II patients included in the MATCH study cohort are reviewed studying the completeness of the description of these 5 factors. In 69.1% of the reports, 1 or more factors were not described adequately. T- stage and N-stage were reported in all cases. Nevertheless, in the 44 Nx patients, the amount of lymph nodes being below 10, was not mentioned being a risk factor and in 2% of the records tumor differentiation was not reported. The presence or absence of LVI was not mentioned in 62.8% of the cases. After 2012 the reporting of LVI became mandatory and was reported significantly

more (33.5% *vs*. 87.5%; $p < 0.001$). Median follow-up of the cohort was 72.4 months (IQR 62.8-80.8). The 1-, 3- and 5-years survival was 98%, 89.1%, and 80.4% respectively. When examining high-risk factors more closely in our cohort of stage II patients we found that these risk factors did not seem to have a significant impact on survival. Also, we did not find a significant association between the failure to report any of the five factors and overall survival. In multivariable analysis, age, moderately- and well differentiated tumors were significantly associated with overall survival.

We can conclude that more accurate pathology reports are needed to describe the five high-risk factors in stage II colon cancer in order to enable proper patient selection for additional treatment.

In chapter 3, we studied the quality of the collected fresh frozen (colon)tumor samples within the MATCH study. We randomly selected 10 tissue samples per participating hospital, excluding samples of patients who underwent neoadjuvant chemo- and/or radiotherapy. The RNA integrity number (RIN) of 70 samples was determined to evaluate the quality (range, 1(low quality) to 10(high quality))[15,16]. The median RIN value of all samples was 7.3 (range, 2.9 to 9.0). A RIN value ≥ 6, which is the cut off value for good quality, was present in 91% of the samples. The median RIN value of the university hospital (a center with large experience in tissue storage) was 7.7. The RIN value of the non-university teaching hospitals ranged from 6.5 to 7.8. The overall median RIN of the non-university teaching hospitals did not differ significantly from the median RIN of the university hospital ($p= 0.39$). These data show that the collection of high quality fresh frozen samples of CRC is feasible in a multicenter design.

Follow-up of patients after resection of the primary colorectal tumor is indicated to detect metastases in an early stage, enabling optimal treatment. However, follow-up programs are expensive and time consuming for patients as well as hospitals. The availability of a biomarker that would allow an out-of-hospital control of the course of the disease would offer a benefit to patients and health care organizations. In search for a marker enabling identification of early metastases, we explored the urine proteome of patients with colorectal liver metastases (CLM). In **chapter 4**, the urine sample preparation and urine sample analyses by mass spectrometry are described. The urine proteome of 24 patients with CLM was compared to that of 25 healthy persons (kidney donors). Seven peptides were discovered that could discriminate between the 2 populations with a sensitivity of 84.6% and a specificity of 92.3%. Additional analyses showed 2 peptides of the seven to be suitable for further research, AGP(-OH)GEAGKP(-OH)GEQGVP(-OH)GDLGAP(-OH)GP and KGNSGEP(-OH)GAPGSKGDTGAKGEP(-OH)GPVG. These peptides combined had a sensitivity and specificity of 69.2% and 84.6% respectively, for discrimination between the two study groups.

CRC blood biomarker CEA (carcinoembryonic antigen) has a sensitivity of approximately 64%[17], therefore additional liver imaging is necessary. In an attempt to improve the detection of CLM we combined the blood CEA test with the urine AGP biomarker **(chapter 5)**. We compared the CEA blood levels and AGP urine levels between 100 patients with CLM and 100 healthy kidney donors. A multivariate logistic regression model was build resulting in a combined sensitivity and specificity of CEA and AGP of respectively 85% and 84%. Further research is needed to evaluate this combined biomarker. Ultimately, a test may be constructed in which the urine AGP test and serum CEA test is combined successfully.

In case of early detection of liver metastases improvement of treatment modalities is an important issue to be addressed. The use of neoadjuvant chemotherapy and minimally invasive therapies for recurrence of CLM are increasingly incorporated in the surgical strategy, allowing proper patient selection, reducing collateral damage and saving remnant liver volume[7-13]. In **chapter 6** we compared two surgical approaches, anatomical (AR) versus non- anatomical liver resection (NAR). The study included 201 patients, 113 patients (56.2%) received an AR and 88 patients (43.8%) received a NAR. Analyzing the data, we learned NARs were performed for significantly smaller metastases (3cm *vs*. 4cm, p=<0.001), received significantly less blood transfusions (20% *vs*. 36%, p=0.012) and had a shorter hospital stay (7 *vs*. 8 days, p=<0.001) compared to AR. There was no significant difference in complications, the rate of positive surgical margins and recurrence rate. For the total study group estimated 5-year disease-free and OS was 31% and 44%, respectively, with no difference between the groups. These results underline NAR to be a save procedure to treat CLM and preserve liver parenchyma.

Unfortunately, liver resection for CLM knows a high recurrence rate[14]. Identifying patients at high risk for disease recurrence, could spare them an operation and might be offered chemotherapy to prevent extension of disseminated disease. To address this problem, we investigated whether the enumeration of circulating tumor cells (CTCs) could identify patients developing disease recurrence within 1 year after liver metastasectomy. The Cell Search Technique is the only method approved by the US Food and Drug Administration. The standard blood sampling volume is 7.5 ml[18]. Several studies concluded that the amount of CTCs retrieved by the Cell Search system in limited CRC disease is very low[18-22]. Therefore, we conducted a pilot study **(chapter 7)** comparing the number of CTCs detected in 7,5 ml peripheral blood to 30 mL peripheral blood prepared by a modified Ficoll density gradient enrichment step in 15 patients. In 7,5 ml blood the median number of CTCs was 1 (range 0 to 4). In 30 ml blood, the median number of CTCs was significantly higher (median 2; range, 0 to 9; p=0.03). Analyzing 7,5 ml blood,

≥ 1 CTC was found in 10 patients (67%) and ≥3 CTCs were found in 2 patients (13%). In 30 ml ≥ 1 CTC and ≥3 CTCs were detected in 13 (87%) and 7 (47%) patients, respectively.

This sample preparation method was used in combination with the Cell Search System in a large study, including 151 patients with CLM, undergoing liver resection **(chapter 8)**. One hundred and seventy- three samples were analyzed. In 75 samples (43%) CTCs were detected, 16% had ≥ 3 CTCs/7,5 ml blood. Eighty-two patients experienced disease recurrence. The 1-year recurrence rate between patients with or without detectable CTCs was similar (47% *vs*. 48%), the presence of a low or high CTC count (<3 or ≥ 3 CTCs/7,5 ml) (50% *vs*. 47%) made no difference. Disease-free and overall survival were similar between patients with or without CTCs. From this study we concluded, that the presence of CTCs in peripheral blood samples does not identify patients at risk for early disease recurrence after curative liver resection for CLM.

In conclusion, the studies presented in this thesis, were conducted with the intention to improve identification of patients at high risk for disease recurrence after colorectal tumor resection as well as after colorectal liver metastases.

On a clinical level, we can state that the completeness of pathology reports can be improved to identify high risk stage II patients. It's an essential step in optimizing patient's prognosis and inclusion for adjuvant chemotherapy.

In addition to the classical pathology report, molecular analysis of CRC may help to identify high risk patient groups. Large cohorts will be needed to unravel the molecular subtypes playing a role in tumor progression. We demonstrated the feasibility of using fresh frozen samples from a multicenter cohort study to allow molecular research, including DNA, RNA and proteomic assays.

To date, research on biomarkers identifying patients with progressive disease is making great steps forward. Focus on urine and blood analysis may reveal new biomarkers within the near future, but large numbers of patients will be needed to allow proper discovery and validation research. With the introduction of the Dutch platform for CRC this research can be facilitated allowing more successful diagnostics and treatment of patients with colorectal cancer.

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NEDERLANDSE SAMENVATTING

Darmkanker is een van de meest voorkomende vormen van kanker. Jaarlijks worden wereldwijd 1.36 miljoen mensen gediagnosticeerd met darmkanker en overlijden 694.000 mensen aan de gevolgen van deze ziekte[1]. Afstandsmetastasen zijn de belangrijkste oorzaak en komen het meest voor in de lever. Voor de bepaling van het ziektestadium wordt gebruik gemaakt van het TNM (tumor, lymfklier, metastase) systeem[2]. Het ziektestadium wordt mede gebruikt om een indruk te krijgen over de prognose en de juiste behandelingsstrategie te bepalen. Volgens (inter)nationale richtlijnen worden patiënten met stadium II ziekte (darmkanker zonder lymfkliermetastasen) niet aanvullend behandeld met chemotherapie. Toch weten we dat 25-30% van deze groep patiënten te maken zal krijgen met metastasen[3]. Patiënten met stadium III ziekte (darmkanker met lymfkliermetastasen) worden wel behandeld met aanvullende chemotherapie. Helaas heeft maar een klein gedeelte van deze groep (5-15%) baat bij deze therapie. Jammer genoeg krijgt wel de hele groep te maken met de toxische bijwerkingen van deze therapie[4]. Vooralsnog kunnen we patiënten met een hoog risico op ziekte recidief niet op een goede manier identificeren. De enige mogelijkheid die geboden kan worden is een regelmatige controle volgens een standaard schema middels bloed- en beeldvormend onderzoek van de lever[5,6]. Deze follow-up is duur en belastend voor patiënten, maar waardevol omdat (lever)metastasen in een vroeg stadium ontdekt kunnen worden en een curatieve behandeling kan worden aangeboden.

Door verbeteringen in chirurgische technieken, aanvullende modaliteiten, zoals ablatie en bestraling, en effectievere vormen van chemotherapie komen meer patiënten in aanmerking voor een curatieve leverresectie[7-13]. Helaas ligt met 50% het 1-jaars recidief percentage erg hoog[14]. Daarom is het belangrijk testen te ontwikkelen die kunnen bepalen welke patiënten een hoog risico op ziekte recidief hebben. Bovendien zouden we geïnformeerd moeten zijn over de kans op een goede reactie bij behandeling, hetzij door operatie, door chemotherapie of een combinatie van behandelingen.

In dit proefschrift worden onderzoeken gepresenteerd naar klinische factoren en biomarkers die de ziekte uitkomsten van individuele patiënten met darmkanker en levermetastasen beter kunnen voorspellen.

Momenteel wordt het TNM-systeem gebruikt om het ziektebeloop van patiënten te voorspellen[2]. De informatie die nodig is voor het vaststellen van het ziektestadium wordt verkregen uit het pathologieverslag. In deze verslagen worden 5 factoren vermeld (tumor stadium, tumor differentiatie, lymphovasculaire invasie (LVI), tumorperforatie en lymfklier metastase) die patiënten met stadium II ziekte en een hoog risico op ziekte recidief kunnen identificeren. In deze groep zou chemotherapie overwogen kunnen worden[3]. Het is dus van groot belang dat deze factoren op een juiste manier worden

beschreven in de pathologie verslagen, aangezien dit van belang kan zijn voor de therapie keuze. In **hoofdstuk 2** hebben we 356 pathologie verslagen beoordeeld van patiënten met ziektestadium II die deelnamen aan de MATCH-studie. In de verslagen hebben we bestudeerd of de 5 factoren op juiste wijze werden beschreven. In 69,1% van de verslagen ontbrak een adequate beschrijving van 1 of meer van de factoren. Tumorstadium werd in alle verslagen juist beschreven; in 44 verslagen werd door ons vastgesteld dat het aantal onderzochte lymfklieren kleiner was dan 10, een aantal dat is gedefinieerd als betrouwbare grens om de lymfklierstatus te benoemen, maar dit was niet expliciet in het oorspronkelijke verslag vermeld. In 2% van de verslagen werd de tumordifferentiatie niet beschreven. Over de aan- of afwezigheid van LVI werd in 62,8% niet gerapporteerd. Vanaf 2012 werd de beschrijving van LVI verplicht gesteld en wij stelden vast dat sinds dat moment de LVI-status ook significant vaker beschreven werd (33,5% versus 87,5%; $p<0,001$). De mediane follow-up van het bestudeerde cohort was 72,4 maanden (interkwartielafstand 62,8 - 80,8 maanden) met een 1-, 3- en 5-jaarsoverleving van respectievelijk 98%, 89,1% en 80,4%. Wanneer de hoog risico factoren nader werden bekeken, vonden we geen associatie tussen de overleving en het niet beschrijven van 1 van de factoren. Uit de multivariaat analyse bleek dat leeftijd, goede - en matige differentiatie invloed hadden op de overleving. Deze resultaten geven aan dat de 5 hoog risico factoren beter beschreven moeten worden in de pathologieverslagen zodat er een optimale patiënten selectie gemaakt kan worden voor verdere behandeling.

In **hoofdstuk 3** hebben we de weefselkwaliteit getest van ingevroren tumorsamples die zijn verzameld in het kader van de MATCH-studie. Per deelnemend ziekenhuis werden willekeurig 10 tumorsamples geselecteerd. Weefsels werden geëxcludeerd indien ze blootgesteld waren aan neoadjuvante chemo- en/of radiotherapie. Om de kwaliteit te beoordelen werd van 70 weefselsamples het RNA-integriteit nummer (RIN) bepaald (schaal 1(lage kwaliteit) tot 10 (hoge kwaliteit)[15,16]. De mediane RIN van alle weefselsamples was 7,3 (uitersten 2,9 -9,0). In 91% van de weefselsamples was de RIN-waarde $\geq$ 6, een waarde die wordt gebruikt als ondergrens voor een goede kwaliteit. De mediane RIN van het universitaire ziekenhuis (een centrum met grote expertise in weefsel opslag) was 7,7. De mediane RIN-waarde van de niet-universitaire ziekenhuizen lag tussen de 6,5 en 7,8. De mediane RIN van alle niet-universitaire ziekenhuis tezamen was 7,3, en deze waarde verschilde niet van de mediane RIN-waarde van het universitaire ziekenhuis ($p= 0.39$). Deze resultaten tonen aan dat binnen een multicentrische studie, waarin ook ziekenhuizen participeren waar geen traditie bestaat in weefselopslag, weefsels verzameld kunnen worden met een hoge kwaliteit.

Follow-up van patiënten na resectie van de primaire darmtumor is geïndiceerd om afstandsmetastasen in een vroeg stadium op te kunnen sporen zodat een optimale behandeling kan worden geboden. Deze follow-up programma's zijn duur en belastend

voor zowel ziekenhuizen als voor de patiënt. Beide groepen zouden baat hebben bij biomarkers die ziekte recidief op eenvoudige en betrouwbare wijze zouden kunnen detecteren, bij voorkeur in een setting buiten het ziekenhuis. In een zoektocht naar een biomarker die levermetastasen (LM) in een vroeg stadium zou kunnen aantonen hebben we onderzoek gedaan naar eiwitten in de urine van patiënten met LM. In **hoofdstuk 4** worden de urine preparatie en de massa spectrometrie analyse beschreven van dergelijke monsters. We hebben het urine eiwitspectrum van 24 patiënten met LM vergeleken met dat van 25 gezonde personen (nierdonoren). Er werden 7 peptiden gevonden die de twee groepen van elkaar konden onderscheiden met een sensitiviteit van 84,6% en een specificiteit van 92,3%. Aanvullende analyses toonden dat 2 van de 7 peptiden geschikt waren voor verder onderzoek, AGP(-OH)GEAGKP(-OH)GEQGVP(-OH)GDLGAP(-OH)GP and KGNSGEP(-OH)GAPGSKGDTGAKGEP(-OH)GPVG. Samen hebben deze twee peptiden een sensitiviteit en specificiteit van respectievelijk 69,2% en 84,6% voor het onderscheiden van de twee groepen.

Darmkanker tumormarker CEA (carcinoembryonic antigen) heeft een sensitiviteit van circa 64%[17], daarom is het noodzakelijk om naast bloedonderzoek ook afbeeldend onderzoek van de lever te verrichten. In een poging om de detectie van LM te verbeteren hebben we de bloedtumormarker CEA gecombineerd met de urinemarker AGP **(hoofdstuk 5)**. We hebben de CEA-waarde en de AGP-waarde vergeleken van 100 patiënten met LM met die van 100 nierdonoren. Voor de analyse werd een multivariaat logistisch regressie model opgesteld, wat resulteerde in een gecombineerde sensitiviteit en specificiteit van CEA en AGP van 85% en 84%. Verder onderzoek is noodzakelijk om deze gecombineerde biomaker te evalueren. Het ultieme doel is een combinatietest te ontwikkelen, die buiten het ziekenhuis toegepast kan worden.

Vroege detectie van LM is van groot belang, maar daarnaast is onderzoek naar de verbetering van behandelmethoden ook een belangrijk onderwerp. Het gebruik van neoadjuvante chemotherapie en minimaal invasieve technieken voor de behandeling van recidief LM worden steeds meer geïncorporeerd in de chirurgische behandelstrategie, waardoor er een betere patiënten selectie kan worden gemaakt, minder additionele weefselschade wordt aangericht en het volume resterend leverweefsel vergroot kan worden[7-13]. In **hoofdstuk 6** hebben we twee chirurgische resectietechnieken met elkaar vergeleken, de anatomische leverresectie (AR) met de niet-anatomische leverresectie (NAR). In de studie werden 201 patiënten geïncludeerd, 113 (56,2%) patiënten ondergingen een AR en 88 (43,8%) patiënten een NAR. De resultaten toonde dat een NAR werd uitgevoerd bij significant kleinere metastasen (3cm versus 4cm, p=0,001), deze patiënten significant minder bloedtransfusies ontvingen (20% versus 36%, p=0,012) en de ziekenhuisopname significant korter was (7 versus 8 dagen, p=0,001) in vergelijking met een AR. Er was geen significant verschil in complicaties, ook het percentage positieve

resectiemarges en het recidiefpercentage was niet verschillend. De 5-jaarsziektevrije en algehele overleving voor de totale studiepopulatie was 31% en 44%, er was geen verschil tussen de 2 studiegroepen. Wanneer we naar deze resultaten kijken, kunnen we stellen dat een NAR een veilige procedure is voor de behandeling van LM waarmee leverweefsel kan worden behouden.

Helaas kent de chirurgische resectie van LM een hoog recidiefpercentage[14]. Wanneer we deze patiënten met een grote kans op recidief op voorhand zouden kunnen identificeren, zouden we deze patiënten een operatie kunnen besparen en eventueel met systemische chemotherapie kunnen behandelen. In een poging patiënten met ziekte recidief binnen 1 jaar na operatie te identificeren, hebben we onderzocht of de aanwezigheid van circulerende tumorcellen (CTCs) hierbij kon helpen. De Cell Search techniek is een methode die goedgekeurd is in Amerika door de FDA (food and drug administration). Bij deze techniek wordt 7,5ml bloed geanalyseerd. Meerdere studies hebben gerapporteerd dat het aantal CTCs in patiënten met beperkte ziekte (alleen aanwezigheid van de darmtumor en/of metastasen in de lever) erg laag is wanneer het Cell Search systeem wordt gebruikt[18-22]. Daarom hebben we een pilotstudie uitgevoerd **(hoofdstuk 7)** waarin we van 15 patiënten het aantal CTCs in 7,5ml perifeer bloed hebben vergeleken met 30ml perifeer bloed wat voorbewerkt is met een Ficoll dichtheidsgradiënt verrijkingsstap. In 7,5ml bloed was het mediane aantal CTCs 1 (uitersten 0 tot 4). In 30ml bloed was het mediane aantal CTCs significant hoger (mediaan 2; uitersten 0 tot 9; p=0,03). Wanneer we naar de 7,5ml bloedsamples kijken, werden er $\geq$ 1 CTC gevonden in 10 patiënten (67%) en $\geq$ 3 CTCs in 2 patiënten (13%). In 30 ml bloed werden $\geq$ 1 en $\geq$ 3 CTCs gevonden in respectievelijk 13 (87%) en 7 (47%) patiënten. Deze sample preparatie methode werd in een grote studie gebruikt, waarin 151 patiënten met LM werden geïncludeerd die een leverresectie ondergingen. In totaal werden 173 bloedsamples geanalyseerd. In 75 samples (43%) werden CTCs gedetecteerd,16% bevatte $\geq$ 3 CTCs. Ziekte recidief werd geconstateerd in 82 patiënten. Het 1-jaars recidiefpercentage was gelijk voor patiënten mét en zonder detecteerbare CTCs (47% versus 48%), de aanwezigheid van een laag of hoog CTC aantal (<3 CTCs or $\geq$ 3 CTCs) maakte geen verschil (50% versus 47%). Tevens was er geen verschil te zien in ziektevrije en algehele overleving tussen patiënten mét en zonder CTCs. Kijkend naar deze resultaten kunnen we concluderen dat patiënten met een hoog risico op recidief ziekte na resectie van LM niet geïdentificeerd kunnen worden aan de hand van CTCs in perifeer bloed.

De studies beschreven in dit proefschrift hadden tot doel om de identificatie van patiënten met een hoog risico op ziekte recidief zowel na resectie van de primaire darmtumor als na resectie van LM te verbeteren.

Ten aanzien van de kliniek kunnen we stellen dat er ruimte is voor verbetering in de beschrijving van de pathologie verslagen, zodat hoog risico ziektestadium II patiënten beter geïdentificeerd kunnen worden aan de hand van klassieke criteria. Dit is een essentiële stap in het schatten van de prognose van patiënten en voor de selectie van patiënten voor aanvullende therapie. Naast het klassieke TNM-systeem, zullen aanvullende moleculaire analyse van darmtumoren nodig zijn om hoog risico patiënten op te sporen. Grote cohortstudies, zoals de MATCH-studie, zijn nodig om de verschillende moleculaire subtypes en hun rol in ziekteprogressie op te helderen. We hebben aangetoond dat het mogelijk is om binnen deze grote cohortstudies een weefselbank aan te leggen met weefsels van goede kwaliteit die gebruikt kunnen worden voor moleculaire experimenten, waaronder DNA, RNA en proteomic analyses.

In de afgelopen tijd zijn er grote stappen gezet in het onderzoek naar biomarkers voor de identificatie van patiënten met ziekteprogressie. In de nabije toekomst kunnen urine, bloed- en weefselanalyses mogelijk nieuwe biomarkers aantonen. Het is echter essentieel dat naast discovery onderzoek validatie plaatsvindt in grote populaties om de waarde voor de klinische bruikbaarheid aan te tonen. Na het MATCH-project is er met de introductie van het Nederlandse platform voor darmkanker, een organisatie gerealiseerd die dergelijke studies kan faciliteren en die de diagnostiek en behandeling van patiënten met darmkanker kan verbeteren.

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Chapter 10

Future perspectives

Colorectal cancer (CRC) is a heterogeneous and molecularly complex disease. Over the years, a clear need has emerged for markers that add clinically relevant prognostic and predictive information to the classic TNM (tumor, node, metastases) staging system to refine the individual patient outcome.

This chapter addresses the problem of tumor heterogeneity, one of the major challenges in cancer research. We also highlight promising research tools and biomarkers and discuss new research strategies and the impact of CRC screening.

TUMOR HETEROGENEITY

Tumor heterogeneity represents an ongoing challenge in the field of cancer therapy. Heterogeneity is evident among cancers from different patients (inter-tumor heterogeneity) and within a single tumor (intra-tumor heterogeneity).

Inter-tumor heterogeneity is explained in part by differences in disease development. The traditional adenoma–carcinoma sequence is believed to be responsible for ~50–60% of CRCs. Other disease-development routes, such as the serrated pathway characterized by serrated adenomatous lesions that frequently display *BRAF* mutations[1] and colitis-associated CRC development with *TP53*[2,3], are thought to account for the other CRC portion. Understanding the various developmental routes of CRC is critical because the different pathways directly affect the clinical course of the disease.

A tumor is a heterogeneous population of cells, consisting of transformed cancer cells, supportive cells, and tumor-infiltrating cells. The nature of this heterogeneity is not limited to this malignant cancer cell population because a tumor is a complex system containing cancer cells and other types such as endothelial cells, stromal cells, and infiltrating immune cells and a complex network of extracellular matrix that is accountable for variations in the tumor microenvironment[4,5].

Two models have been suggested to explain intra-tumor heterogeneity. In the clonal evolution model, stochastic mutations in individual tumor cells act as a platform for adaptation and selection for the fittest clones of a tumor. This model explains heterogeneity within a tumor as a result of natural selection. The clones that obtain growth advantage will increase while the clones with less fitness will be outcompeted[6-8]. The second proposed model for explaining intra-tumor heterogeneity is the cancer stem cell (CSC) model, which suggests that only a subset of cancer cells has unlimited self-renewal ability to initiate and maintain tumor growth[9]. Therefore, tumors are structured in a hierarchical manner, comparable to the normal tissue hierarchy supported by healthy stem cells. It is important to realize that this hierarchy in tumor cells is not a one-way route but can be reversible or pliable so that the terminally differentiated cells can also dedifferentiate and regain CSC properties under specific conditions[10,11].

NEXT-GENERATION SEQUENCING

Next-generation sequencing (NGS) technology emerged at the beginning of this century as an alternative to Sanger sequencing[12]. It allows massive parallel sequencing, reduces turnaround time for analysis, and requires a very low input of DNA/RNA, all of which are major advantages. This technology has multiple applications: whole-genome sequencing covers the complete genome of a sample whereas whole-exome sequencing is restricted to the coding regions (i.e., all exons). Targeted sequencing uses target-enrichment methods to capture and/or amplify regions of interest. RNA sequencing facilitates the detection of alternative gene-spliced transcripts, posttranscriptional modifications, gene fusion, mutations/single nucleotide polymorphisms and changes in gene expression. The introduction of NGS technology in daily practice is hampered by use of multiple sequencing platforms, high-complexity workflow and results, and challenging bioinformatics analysis.

The CRC subtyping consortium used NGS data to investigate inter-tumor heterogeneity, identifying four molecular subtypes of CRC[13]. Data from six independent studies, including 4151 patients, were analyzed. These data were produced on different NGS platforms, and DNA was extracted from fresh-frozen samples as well as from formalin-fixed paraffin embedded (FFPE) materials. Consensus molecular subtype (CMS) 1 was found in 14% of samples, showed hypermutations, and was microsatellite instable and associated with increased expression of genes linked to immune infiltrate. CMS2 was present in 37% of samples and displayed epithelial differentiation, chromosomal instability, and activation of WNT and MYC signaling. CMS3 was discovered in 13% of samples and distinguished by epithelial differentiation and metabolic dysregulation. Finally, CMS4 was identified in 23% of samples and showed upregulation of genes involved in epithelial–mesenchymal transition, prominent transforming growth factor β activation, stromal invasion, and angiogenesis. Of the samples analyzed, 13% showed mixed features of the four CMS subtypes. The subtypes were also associated with clinical and prognostic factors; for instance, CMS1 was frequently diagnosed in women with right-sided lesions and high histopathological grade whereas CMS2 tumors were mainly left-sided, and CMS4 tumors tended to be diagnosed at a more advanced stage (III-IV). Concerning prognosis, CMS4 tumors displayed worse disease-free and overall survival. CMS1 patients had very poor survival after relapse, in contrast to CMS2 patients, who showed superior survival after relapse.

Furthermore, NGS enables the identification of biomarkers and targets for therapies, with more accuracy and specificity than traditional sequencing methods. Thus, NGS is a valuable instrument in the unraveling of CRC biology, improving research quality and care for CRC patients.

PROMISING BIOMARKERS

The liquid biopsy

A potent approach for biomarker discovery is the liquid biopsy, a minimally invasive process based on a simple venipuncture that has multiple advantages: it is safe, implemented on a wide scale, and can be repeated easily. Moreover, through a liquid biopsy, the disease course can be monitored, and molecular changes in the tumor can be detected over time. Biomarkers that can be a subject of interest in a liquid biopsy can be protein-based, such as cancer antigens (carcinoembryonic antigens (CEA)); cell-based, such as circulating tumor cells (CTCs) and disseminated tumor cells; or nucleic acid–based, such as circulating free DNA (cfDNA) and microRNAs (miRNAs).

Circulating tumor cells

Primary tumors begin shedding neoplastic cells into the circulation at an early stage[14,15], and approximately 10^6 cells are shed daily per gram of tumor[16]. CTCs constitute a heterogeneous population of cells with different biological characteristics and are often different from the primary tumor. Because CTCs are usually present in very low frequencies in peripheral blood, tumor cell enrichment techniques are used before applying detection methods, including density gradient centrifugation (Ficoll-Hypaque separation) and immunomagnetic or size filtration procedures[17,18]. Different detection methodologies are in use, such as the more labor-intensive PCR techniques, the less time-consuming semi-automated CellSearch Technique (Veridex LLC, Raritan NJ, USA), membrane arrays, and weighted enzymatic chip array. The large majority of these techniques depend on monoclonal antibodies targeting epithelial cell adhesion molecule (EpCAM) for the isolation of CTCs. It has been reported that in tumor cells that have gained entry to the bloodstream, EpCAM expression is 10-fold less compared to primary and metastatic tissues[19]. This difference is due to the microenvironment and to EMT. During EMT, tumor cells lose their epithelial traits including cell–cell adhesion, apical–basal polarity, and lack of motility and acquire mesenchymal properties such as motility, invasiveness, and a resistance to apoptosis[20]. Therefore, alternative isolation methods are now being developed that avoid the use of antibody-based enrichment. In spite of different detection methods, CTCs have proved to be of prognostic and predictive value. Investigations by Uen et al. and Lu et al. demonstrated that the persistent presence of postoperative CTCs is a poor prognostic factor for patients with CRC after curative resection[21,22]. The CellSearch Technique has been approved by the US Food and Drug Administration to evaluate chemotherapy response by CTC counting in patients with metastatic CRC[23]. Likewise, the *KRAS* mutation status of CTCs can be used to predict therapy response in patients treated with cetuximab and FOLFOX4 or FOLFIRI[24,25].

Circulating free tumor DNA (cfDNA)

cfDNA may originate from normal or tumor tissue and be present in increased levels in noncancerous conditions such as inflammatory processes and infections[26]. DNA fragments can be passively released by necrotic and apoptotic cells, depending on the tumor load, growth kinetics, and the effect of antitumor treatment. It has also been reported that tumors actively release cfDNA that can transform cells at distant sites[27]. Finally, CTCs and micrometastases may also be a source of cfDNA. The short half-life of cfDNA (~2 hours) makes cfDNA a useful dynamic marker of tumor bulk[28]. Unfortunately, because of a lack of standardized techniques and low concentrations of cfDNA, identification, amplification, and quantification are challenging. Moreover, selection of the proper mutation markers for cfDNA analysis is a difficult issue[29]. In spite of these obstacles, multiple studies have been conducted to investigate if cfDNA can be used as a biomarker in CRC.

The presence of minimally residual and invasive disease can be identified by cfDNA. In a study by Reinert et al.[30], the quantification of plasma cfDNA had almost 100% sensitivity and specificity for the prediction of relapse after surgery, with a mean lead time of 10 months. Tie et al.[31] demonstrated that stage II colon cancer patients who were cfDNA-positive postoperatively were at extremely high risk of recurrence (hazard ratio=18; 95% confidence interval, 7.9–40; $p \leq 0.001$). cfDNA has also been shown to be useful in the early detection of relapse after metastasectomy of liver metastases, significantly outperforming both CEA and imaging[32]. For the prediction[33,34] and monitoring[35,36] of therapy, as well as the detection of therapy resistance[37-39], cfDNA is under investigation.

MicroRNAs (miRNA)

miRNAs are a group of noncoding RNAs containing approximately 18–25 nucleotides that affect posttranslational gene expression and can be detected in serum, plasma, and tissue samples. Hundreds of genes in the human genome encode these RNAs [40-42], and an estimated ~30% of protein expression is controlled by miRNAs[43,44]. Numerous studies have revealed several important roles in many biological functions for miRNAs, including in proliferation, differentiation, development, and metabolism.

A large number of miRNAs have been described as correlated with CRC. For the most part, these miRNAs influence the main signaling pathways in CRC, such as the WNT[45-49], EGFR[50-55], TP53[56-59], and transforming growth factor beta (TGF-β) pathways[60-62].

The detection of miRNA in plasma can enable early detection of CRC. miR-92a is reported to differentiate CRC and advanced adenomas from other colorectal-related disease[63]. Furthermore, stool could be a source of CRC-specific miRNA. Higher levels of miR-21 and miR-106a have been detected in feces of patients with CRC and advanced adenomas compared to healthy individuals[64]. miRNAs also can be of use for monitoring minimal residual disease and recurrence. Plasma levels of miR 17-3p and miR 92a are

raised in patients with CRC and decrease after surgical removal of the primary tumor[63]. Monitoring of treatment response by means of miRNAs has been investigated, and Let-7g and miR-181b expression levels are strongly associated with the drug chemoresponse on S-1,5-fluorouracil–based antimetabolite[65].

THE CLINICAL APPROACH

Cohort studies and biobanking

Because of the increasing subclassification, availability, and need for validation of new promising biomarkers, and the new technologies and interventions for CRC, there is a need for novel clinical trial designs, methods for data acquisition, and patient recruitment. Cohort multiple (cm) randomized controlled trials (RCTs) are an innovative alternative to the classic RCT, combining features of a prospective cohort study with the possibility of including patients from the cohort in multiple non-conflicting cohort studies[66]. The purposes of this design are to facilitate research, include more patients in RCTs, enable easy selection of subgroups, and generate results that can be extrapolated to the general population. Because of its small size, excellent infrastructure, and high-quality health care system, the Netherlands is a country that can excel in this study approach.

The Dutch Colorectal Cancer Group has launched a cmRCT called the "prospective Dutch ColoRectal cancer cohort" [Dutch: "Prospectief Landelijk ColoRectaal kanker Cohort" (PLCRC)]. In this project, extensive observational clinical data are collected as well as patient-reported outcomes. For each included patient, fresh-frozen and FFPE samples from tumor and normal colon tissue are stored. Additionally, the collection of blood samples is also approved in this national initiative. With this research approach, we hope to improve treatment outcomes in CRC patients[67].

CRC screening

Probably the most powerful instrument for improvement of CRC outcomes is the early detection of CRC and advanced adenomas. Multiple strategies are available for CRC screening, including fecal occult blood testing (with the use of guaiac-based or immunochemical tests)[68-73], alone or in combination with stool DNA examination [74], endoscopy (flexible sigmoidoscopy or colonoscopy)[75-77], radiologic examination (computed tomographic colonography)[78], and testing for blood-based molecular markers, such as circulating methylated septin 9 gene DNA[79]. Multiple high-quality studies support a strategy of fecal occult blood testing every year or every 2 years, with colonoscopy used as a follow-up to a positive test to screen for CRC. Several RCTs have reported mortality reduction even up to 32% with a follow-up[69-73].

In 2014, a screening program for CRC was introduced in the Netherlands for persons ages 55 to 75 years. Biannually, immunohistochemical fecal occult blood testing (iFOBT) is performed, followed by a colonoscopy when the test is positive. With a hypothesized response rate of 60%, it is estimated that 1400 CRC deaths could be prevented[80]. In 2014, a total of 703,626 persons were invited; the response rate was 71.3%, and the test was positive in 40,842 cases (7.8%). In this group, colonoscopy identified 2483 individuals with CRC and 12,030 with advanced adenomas[81]. The detection and treatment of the advanced adenomas may result in a decrease in CRC incidence in the near future, and the identification of asymptomatic CRC patients with lower tumor stages is expected to improve disease-free and overall survival.

THE FUTURE

Implementation of screening programs is of utmost importance and the most effective strategy to reduce CRC incidence and improve CRC survival rates. Once patients are diagnosed with CRC, they should be included in cmRCTs because more patients are eligible for participation in RCTs, which may offer enhanced survival. The availability of biomaterials in this study design facilitates biomarker research, discovery studies, and validation studies. These biomarkers can be identified in different levels of the cancer genome; as discussed above, CTCs, miRNAs, and cfDNA are promising biomarkers and should be investigated in more depth. NGS may help in this mission because it is more accurate, less labor intensive, and faster than other methods. Adding this information to the classical TNM staging system may help refine prognosis, guide treatment strategies for individual patients, and improve CRC outcomes.

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Appendices

Dankwoord

List of publications

PhD portfolio

Curriculum Vitae

DANKWOORD

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LIST OF PUBLICATIONS

Nonphysician clinicians in the follow up of resected patients with colorectal cancer.
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(Accepted for publication Digestive Diseases)

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J Gastrointest Surg 2009;13(5):890-95.

PHD PORTFOLIO

Name PhD student:	Zarina Shehnaaz Lalmahomed
Erasmus MC Department:	Surgery
PhD period:	January 2007- March 2017
Promotors:	Prof. dr. J.N.M. IJzermans
	Prof. dr. C. Verhoef

	Year	Workload (ECTS)
PHD TRIANING		
General Courses		
Classical methods for data analysis	2009	5.7
Good clinical practice	2009	1.0
English writing course	2009	0.3
Introduction to clinical research	2008	0.9
Biostatistics for clinicians	2008	1.0
In-depth courses		
Course Biomedical Research Techniques	2009	1.0
Basic and Translational Oncology	2007	1.8
National conferences		
Najaarsvergadering (2 oral presentations)	2009, 2014	2.0
Chirurgendagen (oral and poster presentation)	2007, 2014	2.0
SEOHS (oral presentation)	2010, 2013	2.0
International conferences		
ESSR (oral presentation)	2010	2.0
ESMO (2 poster presentations)	2010,2013	2.0
Other		
Presentation/interview MLDS	2012	0.3
Presentation kick-off meeting CONNECTION	2014	0.5
TEACHING ACTIVITIES		
Lecturing		
MATCH-study introduction presentations	2007-2008	2.0

Supervising practicals and excursions		
Examination of Basic Life Support for medical students (coordinator instructors 2008/2009/2010)	2007-2013	2.0
Supervising Master's theses		
D. Koole en B. Zegers	2007-2008	2.0
K. Woltman	2008	2.0
M. Bröker en J. Bekken	2008-2009	2.0
D. van Zanten	2009	2.0
Y. Özbay	2009	2.0

CURRICULUM VITAE

Zarina Shehnaaz Lalmahomed werd geboren op 13 februari 1982 te 's Gravenhage. Na het eindexamen Gymnasium, aan het Interconfessioneel Makeblijde College (IMC) te Rijswijk Z-H in 2000, startte zij met de opleiding Geneeskunde aan de Erasmus Universiteit Rotterdam. De laatste fase van haar coschappen heeft zij voltooid op de afdeling Heelkunde van het IJssellandziekenhuis in Capelle aan de IJssel (opleider: Dr. I. Dawson). Voor haar keuze coschap is zij naar Suriname geweest en heeft gewerkt op de afdeling Heelkunde van het Diakonessen Ziekenhuis in Paramaribo (begeleider: drs. A. Bergen en drs. R. Does). In november 2006 behaalde zij haar artsenbul. In januari 2007 begon zij als arts-onderzoeker op de afdeling Heelkunde van het Erasmus MC onder de begeleiding van Prof. dr. J.N.M IJzermans en Prof. dr. C. Verhoef. Het onderzoek wat gedurende deze aanstelling heeft plaatsgevonden vormt de basis van dit proefschrift. Tijdens deze periode schreef zij meerdere subsidieaanvragen en deze werden gehonoreerd door Fonds NutsOhra, de Maag Lever Darm Stichting, Stichting Coolsingel, Erasmus MC Doelmatigheidsonderzoek en het Koningin Wilhelmina Fonds. In januari 2011 begon zij aan de opleiding tot chirurg in het Erasmus MC (opleider: Prof. dr. J.N.M. IJzermans/ Dr. B.P.L. Wijnhoven). In 2013 onderbrak zij de opleiding om de lopende studies waaraan zij begonnen was tijdens haar onderzoeksperiode af te ronden. In januari 2014 hervatte zij de opleiding in het Franciscus te Rotterdam (opleider: Dr. T.M.A.L. Klem). Zarina is getrouwd met Ronny Kalloe en samen hebben zij een prachtige zoon, Viyan.


Fresh frozen tumor
Lymphnodes Follow up
AGP KRAS CTC's
Liver metastases
Colorectal cancer
EA Urine MSI TNM
Fresh frozen tumor
ow up Collagen type a1: AGP
Lymphnodes CEA