# Microscopical evaluation of prognostic factors in colorectal cancer

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*I have sent you my invitation,*
*the note inscribed on the palm of my hand by the fire of living.*
*Don't jump up and shout, "Yes, this is what I want! Let's do it!"*
*Just stand up quietly and dance with me.*

- Oriah Mountain Dreamer, *The Dance* -

Lieve Pa, voor jou

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# Chapter 1

General Introduction

## 1. Colorectal carcinoma

### 1.1 Epidemiology

Colorectal cancer (CRC) is the fourth most common form of cancer occurring worldwide, with about 1.02 million new cases and an estimated 529,000 deaths per year. In Europe, colorectal cancer is the second most common form of cancer, with more than 375,000 new cases diagnosed each year resulting in 203,000 deaths; corresponding figures for The Netherlands are 10,000 cases and 4300 deaths.[1,2] For the next years the incidence of CRC is expected to keep rising due to an aging population and improved detection methods.

Early detection of CRC considerably improves prognosis as therapy can be given in an early stage.[3] To reduce mortality, population screening is being investigated for early detection. The choices available for CRC screening are FOBT (fecal occult blood testing), immunochemical FOBT, flexible sigmoidscopy every 5 yr, or colonoscopy every 10 yr.[4] CT colography is another option but this technique has not been firmly established yet. Using video capsule endoscopy (VCE) the small intestine which is not accessible by the conventional endoscope can be investigated. In Europe FOBT screening is considered for CRC screening because of its proven efficacy for mortality reduction where similar results of screening with other methods are still awaited.

### 1.2 Staging

The internationally accepted systems for staging and grading of tumors can be used to describe the extent of disease, compare groups of patients and determine optimal therapy, select patients for clinical trials, evaluate results of clinical trials and discuss the prognosis with the patient. The current method for staging of colorectal cancer is according to the TNM (Tumor-Node-Metastasis) classification. TNM is the most widely used system for classifying the anatomic extent of cancer spread and important for decision making in therapy.[5] Information on nodal involvement is an important part of CRC staging since metastasis to regional lymph nodes (LNs) is one of the most important factors relating to the prognosis of colorectal carcinomas. Patients with metastatic LNs have a shorter survival and require adjuvant systemic chemotherapy. Despite this, nodal involvement alone is not considered sensitive enough due to the detection sensitivity or non-lymphogenic spread. Thirty percent of all patients initially diagnosed with node-negative colorectal cancer eventually relapse and die from disseminated disease, showing current staging to be suboptimal.[6]

The five year survival rate for colon cancer stage II patients (AJCC staging) is 85% for stage IIA and 72% for stage IIB.[3] There is controversy in the necessity of adjuvant treatment for all stage II patients as is shown in several studies.[7-11] However chemotherapy might improve survival in the node negative high-risk patients who are likely to have a recurrence.[12]

During the ASCO (American Society of Clinical Oncology) Annual Meeting (June 2–6, 2006, Atlanta, GA) recommendations for treatment of stage II disease were proposed. Experts in GI cancer reported the results of a meta-analysis on 7 randomized trials (3,732 patients) and concluded that there is no rational to routinely apply adjuvant therapy, with the exception of high risk cases based on clinical features (T4,

obstruction or perforation), nodal sampling (number of LNs resected) and high risk prognostic factors.
Prognostic information is of importance for the life expectation of the patient, for the selection of patients for adjuvant treatment schedules and for an intensive follow-up policy. Staging has originally been developed to compare patient groups and treatment results. The current TNM staging system has not been primarily developed for prognosis and is therefore less suitable. This system has met the conditions in the time of "watchful waiting" but in present time with the possibility of adjuvant treatment regimes as adjuvant chemotherapy, radiotherapy, targeted therapies and liver-surgery its shortcomings become clear.
Stage II patients, who are treated with chemotherapy, have a lower risk on recurrence, but systemic treatment of all stage II patients will lead to an unnecessary morbidity.[13] For this reason chemotherapy in stage II patients is not part of the standard treatment regime although various adjuvant options are available. The clinical question therefore evidently is: how can we identify patients who need additional adjuvant therapy and how can we choose the best suitable treatment for the individual patient?
At this moment the value of the TNM classification is called into question. Because of the current early detection of tumors due to screening programs, the size of the tumor has become of less importance. At the European Breast Cancer Conference in Nice 2006 (EBCC-5) there was a debate about the relevance of using the biology of the tumor as a less subjective, independent parameter for prognosis.[14] The consensus of this conference was that information about the tumor biology should be used next to the TNM classification. Notably for breast cancer, biological parameters as ER, PR, HER2 and p53 status are already more informative than the TNM classification as presented at the St. Gallen International Conference in Switzerland 2007.[15]
For colorectal cancer a variety of molecular tumor markers characterized in the laboratories, have been studied in the clinic for their potential to predict disease outcome or response to therapy. However, very few markers appear to provide definitive prognostic or predictive information. The majority of these clinical studies were retrospective and the results so far have not always been identical with significant discordance between detection methods of marker expression. Furthermore validation of well defined series performed in different institutes is lacking.

## 2. Tumor markers

A tumor marker can be defined as a tool which enables the clinician to answer clinically relevant questions regarding a patient's cancer. By definition, a marker represents a qualitative or quantitative alteration or deviation from the norm of a molecule (DNA, RNA, protein), substance or process that can be detected by some type of assay.[16]
In this chapter the different markers available for colorectal cancer are discussed.

### 2.1 Prognostic markers

Prognostic markers for colorectal cancer can be divided in tissue-based markers, biological, genetic and molecular markers. During the "Prognostic Factors Consensus Conference" of the CAP (College of American Pathologists) these markers

have been subclassified in five categories according to the impact of their prognostic value.[14]

*Category I*: with proven prognostic importance; predominantly tissue-based markers as stage T4, low number of removed lymph nodes, perforation or obstruction, resection status, angio-invasion and serum carcino-embryonic antigen (CEA).

*Category II*: of prognostic importance but not yet validated in clinical studies; histological grading, circumferential resection margin (CRM), micro-satellite-instability (MSI), 18q deletions and growth-pattern.

*Category III*: not analyzed substantially to determine the prognostic importance; predominantly biological and molecular factors as DNA ploidy, tumor suppressor and oncogenes, growthfactors, apoptosis- and angiogenesis related genes, cellular proteins (uPA, MUC-1, E-Cadherin, Ki-67), p53 and K-ras mutations, high thymidylate synthase (TS), and some tissue based markers as the occurrence of fibrosis, desmoplastic stroma, inflammation reactions and proliferation activity.

*Category IV*: New factors with unknown prognostic importance.

Next to the above mentioned factors progress has been made in the field of genomic and expression array for the identification of a set of genes to identify patients with a "bad prognosis" profile.[17]

**2.2 Morphological markers**

*2.2.1. Staging and grading of primary tumors.*

Staging describes the tumor outgrowth, local or with distant metastases. According to guidelines of the International Union against Cancer (UICC), and the American Joint Committee on Cancer (AJCC) staging has to be based on clinical parameters at time of diagnosis, including the size (T stage) of the tumor, the loco-regional lymph node status (N stage) and the presence of distant metastases (M status).

Histological investigation of the tumor, after surgery of the tumor, gives a more precise definition of the T, now called pT and status of the lymph nodes (pN).

Grading reflects the morphology and the proliferative capacity of the primary tumor. Microscopical analysis of tissue serves two goals: firstly to decide on the diagnosis cancer and secondly to determine the differentiation grade of the tumor (well-moderate-poor).

*2.2.2. The circumferential resection margin.*

Local recurrence is an important factor for prognosis following curative resection for rectal cancer.[18] For patients with local recurrence after resection for rectal cancer prognosis is worse with a chance on death due to disease of 90%. Not just the histopathological characteristics have impact on survival; also the type of surgery is an important factor. The CRM (circumferential resection margin) has been first described by Quirke[19] and consists of macrosectioning of the complete tumor with intervals of 3-5 mm to detect dissemination of the tumor.

After the introduction of total mesorectal excision (TME), which consists of complete removal of the rectum together with the mesorectum by precise dissection along the mesorectal fascia, local recurrence rates have significantly decreased to below 10%.[20] TME is currently accepted as the standard treatment in rectal cancer surgery.[21,22,23] CRM involvement is related to a high local recurrence and is a powerful prognostic factor. Local recurrence

due to CRM involvement can be further reduced by preoperative radiotherapy with or without chemotherapy than TME alone. The estimation of CRM is based on H&E stained slides but recent research is focusing on the use of preoperative magnetic resonance imaging (MRI) for a more accurate prediction of CRM.[24] The combination of CRM with nodal status has proven to be a better parameter for rectal cancer as compared to the conventional TNM classification.[25]

*2.2.3. Angiogenesis.*

Angiogenesis is an important step in the outgrowth of a primary tumor and also provides a source for haematogeneous dissemination, progression and metastasis. Potential angiogenic factors are VEGF and platelet-derived endothelial cell growth factor (PD-ECGF).[26,27] VEGF is the most important and has been examined for its role in invasion and metastasis of cancer. Colorectal cancers with increased VEGF expression are known to be associated with a poor prognosis.[28]

Vascular endothelial growth factor (VEGF) expresses its effects by binding to two VEGF receptors, Flt-1 and KDR. Flt-1 shows tyrosine kinase activity that is important for the control of cell proliferation and differentiation. Kaplan et al demonstrated that bone marrow derived haematopoietic cells that express VEGF-R1 home to tumor specific premetastatic sites and form cellular clusters before the arrival of tumor cells. They also found that VEGF-RI positive cells express VLA4, also known as integrin alpha-4-beta-1, and that tumor-specific growth factors upregulate fibronectin, a VLA4 ligant, in resident fibroblasts, providing a niche for incoming tumor cells.[29] Orimo et al demonstrated that CAFs (cancer associated fibroblasts) promote angiogenesis by recruiting endothelial progenitor cells (EPCs) into carcinomas, an effect mediated in part by SDF1 (stromal cell derived factor 1).[30]

Angiogenesis is not a pure morphological marker but also a molecular marker.

**2.3 Biological markers**

There is evidence that EMT gives rise to the dissemination of single carcinoma cells from the sites of the primary tumors.[31] Cancer cells can be released from primary tumors in the bloodstream with an estimated $10^6$cells/g of tumor (approx. $10^9$ cells).[32] The presence of cancer cells in local and regional areas (LNs) that surround primary tumors is an indicator of metastasis. Cancer cells are also detected in the blood of patients with known primary tumors, and tumor cells metastasized to the bone marrow have been detected by immunocytochemistry.

The presence of micrometastasis has shown to be an independent prognostic indicator for recurrence and survival.[33, 34] Patients with single disseminated tumor cells in the blood and bone marrow are target groups for adjuvant therapy. These cells often show different properties than cells of the primary tumor, so further molecular analysis unravel molecular “fingerprints” and will help to develop targeted antimetastatic therapies.[35]

However not all circulating tumor cells will survive in the circulation and are therefore functional. Current research focuses on the identification of tumor stem cells responsible for metastases.[36]

There are different routes for tumor cell dissemination; local invasion, haematogeneous routes or lymphatic routes. Sec-

ondary dissemination also occurs from overt metastases to other distant sites.

Various model systems for metastasis were reviewed by Pantel and Brakenhoff.[37] In the first model, disseminated tumor cells settle and proliferate in the lymph nodes to form solid metastases. At later stages, tumor cells disseminate from the established lymph node metastases to distant sites, where they form secondary metastases. According to this model the tumor cells at distant sites die or remain dormant or proliferate.

Haematogenous dissemination may occur from the primary tumor, the lymph node metastases or from distant metastases. In the second model, tumor cells primarily undergo haematogenous dissemination to form distant metastases. This occurs in patients who develop metastases at other organs, whereas the lymph nodes remain tumor free, such as in patients with breast cancer.

Haematogenous dissemination seems to start at the earliest stages in tumor progression, as tumor cells migrated to the bone marrow have been detected in a significant proportion of patients with tumors less than 2 cm in diameter.[38] The accurate detection of the presence of tumor cells in the BM or blood of node negative (stage I,II) patients is therefore a valuable tool in the selection of high-risk patients for adjuvant treatment.

*2.3.1. Detection of tumor cells in lymph nodes.*

Accurate analysis of locoregional lymph nodes (LNs) is of major importance, since it determines the choice of adjuvant therapy for the patient. However, routine histological investigation of LNs only involves one tissue section per node (<1%) and metastases can easily be missed. Usually this concerns groups of cells so called micrometastases (MM) or isolated tumor cells (ITC). MMs are defined as deposits of tumor cells of 2 mm or less but larger than 0.2 mm and ITC either as single tumor cells or as clusters of tumor cells of 0.2 mm or less.

The need to detect these cells has led to a more thorough analysis of the LNs using the reverse-transcriptase polymerase-chain-reaction (RT-PCR) methods and immunohistochemical (IHC) staining procedures. Using IHC, antibodies directed against i.e. cytokeratin are used to visualize the epithelial tumor cells which makes them more easy to detect and available for verification by a pathologist. Expression of cancer related genes as the carcinoembryonic antigen (CEA) can be sensitively detected using RT-PCR. RNA from the LNs is isolated and amplified after synthesis of cDNA. A positive signal indicates the presence of tumor cells in the investigated material.

The presence of micrometastases is of clinical impact.[39] However relatively less is known about the biological meaning of single (isolated) tumor cells, and a controversy exists in literature on the impact of the presence of these cells for patient prognosis.[40, 41]

Since detailed examination of all lymph nodes is valuable but labor-intensive, the sentinel node (SN) procedure has been developed.[42] The most important goal of this procedure for colorectal cancer is to select those lymph nodes which have the highest change for harboring tumor cells. For the SN procedure a blue dye (Patent blue) is injected in vivo around the primary tumor during the operation. After several minutes the first nodes to which the tumor

drains will color blue. In the resected material these nodes will be marked with suture and thus be recognizable for the pathologist. The current SN procedure is considered reliable if the first 2-4 nodes stain blue.

An advantage of this procedure is that these nodes can be more accurately investigated for the presence of micrometastases using above mentioned IHC techniques and multiple sectioning. For colorectal cancer the SN procedure in combination with IHC has a specificity of 97% and a sensitivity of 91%.[43] A recent published first prospective evaluation shows an upstaging from 8% of N0 to N1 and a significant difference in disease free survival for patients with and without presence of micrometastases ($p=0.002$) based on IHC and quantitative PCR.[39]

*2.3.2. Detection of tumor cells in blood and bone marrow.*

One speaks about circulating tumor cells (CTC) when cells are detected in the blood. Tumor cells can also be found in the bone marrow (BM) of patients with primary tumors; they are then called disseminated tumor cells (DTC). Tumor cells in the BM can occur in frequencies as low as 1 cell per $1x10^6$ white blood cells. Therefore enrichment with Ficoll density gradient separation or other enrichment techniques is essential.

Prominent methods used for the detection of CTC's en DTC's are immunocytochemistry (ICC), magnetic cell sorting and RT-PCR. The presence of DTC's in the BM of breast cancer patients has appeared to be an independent parameter for disease free and overall survival and is perhaps more powerful than the presence of positive LNs.[33,34] For CRC it is known that the presence of tumor cells in BM is of prognostic importance while metastases to the bone are sporadically seen. Although several studies have been published reporting the detection of DTC's in BM from CRC patients with RT-PCR, few studies report the prognostic significance.[44] Soeth et al examined BM samples from patients with CRC by using a CK20 nested RT-PCR and report a shorter survival for patients with positive BM.[45] The studies investigating the clinical relevance of DTC's in BM from CRC patients using ICC or magnetic cell sorting reported poorer survival for patients with positive BM.[46-50]

Ongoing research in this field aims to identify more specific markers and to determine the invasive potential of these cells.[51] Are the properties of these single cancer cells identical to the primary tumor? Can we predict their disseminating potential using genotyping? Are they able to form clinically detectable secondary metastases? And how do they home to, and survive in, their target organs?

The biology for CTC's and DTC's differs. Enumeration of the number of circulating tumor cells in blood is considered useful to monitor patients under therapy. Also the number of CTC's in the peripheral blood in patients with stage I-III breast cancer was found to be an independent predictor of progression-free and overall survival, and an increase in the number of CTC's was found to correlate with a fast progression of disease.[52, 53]

**2.4 Automated analysis**

For the detection of occult cells in bone marrow, peripheral blood and lymph nodes nowadays automated imaging devices are available.[54] The detection of the cells (either using fluorescence or bright field

imaging) is performed on basis of color or intensity in combination with artifact rejection routines. Visual confirmation of the selected tumor cells remains the responsibility of the operator or pathologist. Major advantage is that the cell and numerical information are saved for morphological or molecular studies.

Automated analysis of LNs results in a higher detection of occult cells and small tumor groups.[55] In a study performed in two different types of hospitals (academic versus peripheral) respectively 8.5% and 12.5% of breast cancer patients with occult cells or micrometastases present in the LNs were missed by routine histopathological investigation, but were selected using automated microscopy.

The detection of rare tumor cells in BM is a tedious and time consuming task since this frequency can be as low as 1 per $1.10^6$. Automated microscopy can help to find these cells reliably and allows for further investigation of these cells using fluorescence in situ hybridization (FISH) or after laser-capture microscopy, by PCR, array-CGH or expression array.[35]

The Veridex-system by Immunicon has made the analysis of peripheral blood for the detection of circulating cells feasible. This system is based on the selection of tumor cells in whole blood with EpCAM antibodies labeled with ferrofluid, and further verification with markers for cytokeratin (tumor cells) and CD45 (white blood cells).[52, 53]

Recently a promising new technology was introduced to isolate cells from peripheral blood using microchip technology. The described method uses a microfluidic platform mediated by the interaction of CTC's with EpCAM antibody-coated microposts under controlled laminar flow conditions. This chip can identify CTC's in patients with metastatic lung, prostate, pancreatic, breast and colon cancer with 99% sensitivity and a range of 5 to 1,281 cells per ml blood.[56]

**2.5 Molecular markers**

Considering the development of CRC two major pathways are widely accepted. The chromosomal instability pathway (adenoma-carcinoma sequence) including the wnt-signaling pathway,[36] which is characterized by allelic losses, and the other is a pathway involving microsatellite instability (MSI).[57] However, recent publications show that also other routes exist as the TGF-β/SMAD signaling pathway.[58]

The TGF-β/SMAD signaling pathway is composed of TGF-β receptor type I (TGF-β-RI) and type II (TGF-β-RII) and a series of SMAD proteins of which SMAD4 is best described for colorectal cancer as it functions as a tumor suppressor gene. When TGF-β binds to TGF-β-RII, which then complexes with TGF-β-RI, TGF-β-RI phosphorylates SMAD2, which binds to SMAD4. This complex translocates into the nucleus and induces the Cdk inhibitors, p15 and p21, leading to growth arrest. Therefore the loss of SMAD4 function either by mutations or deletions (18q) is of relevance in CRC development.

In addition the transforming growth-factor TGF-ß is an important regulator of the wound healing process.[59] Fibroblasts –the main cell type in stroma- may differentiate into so-called cancer-associated fibroblasts (CAFs) during the progression to invasive carcinoma.[60,61] It also has been suggested that epithelial cells can differentiate into myofibroblasts via TGF-ß dependent epithelial mesenchymal transition (EMT).[62,63]

This EMT represents a fundamental mechanism by which tumor epithelium may disaggregate and reshape for movement into the extracellular matrix. EMT is engaged by several cytokines associated with proteolytic digestion of the basal membrane (by metallo-proteinases) upon which the epithelium resides.

The role of the TGF-β signaling pathway thus relates to both the primary tumor and the stroma. In addition, its role is dual.[64] In the normal colon TGF-β serves as a tumor suppressor pathway by inhibiting cell proliferation and inducing apoptosis. Abnormal function of this pathway may contribute to the initiation and progression of cancer. In early stages of epithelial tumors it blocks tumor growth, whereas in progressed stages it stimulates invasion and metastasis.

Smad proteins are key signal transducers of the TGF-β pathway and are essential for the growth suppression function of TGF-β.[65] Smad proteins act as tumor suppressor molecules whose mutation, deletion, and silencing is associated with many types of cancer. SMAD4, whose gene is coded at chromosome 18q21.1, affects gene transcription and controls cell growth. It is deleted in 30% of invasive and metastatic colon carcinoma.[66-68]

*2.5.1 Genomic and expression profiling using array technology.*

Genomic copy number changes are found frequently in colorectal cancers and are believed to contribute to their development and progression through inactivation of tumor suppressor genes and amplification of oncogenes.

Comparative genomic hybridization (CGH) was developed to allow for genome-wide screening of copy number changes.[69] Gain of chromosome 20q is a widespread finding in primary CRC (67%) as is loss of 18q (49%).[70] Other consistent regions of copy number gain are 7p, 8q, 13q and 12p along with deletions of 8p and 4p. Conventional CGH has a limited resolution and can only detect losses of 10 Mb or greater.[71, 72]

The resolution of CGH has been improved by replacing the metaphase chromosomes as the hybridization target with mapped and sequenced clones (bacterial artificial chromosomes, P1-derived artificial chromosome and cosmids) arrayed onto glass slides. Array-based comparative genomic hybridization (array-CGH) allows for ~ 1Mb or even 5-10 Kb genome-wide screening of DNA copy number changes in solid tumors.[73-75] Copy number alterations detected by array-CHG may aid in the identification, localization and validation of cancer causing genes. Array-based CGH has been applied to a number of colorectal cancer studies and reported small, with CGH undetectable, genomic regions with a number of genes of interest as in particular gain of 17q11.2-q12, 8q24.21 and 8q24.3 and a loss of 4q34-q35.[76, 77]

Although there have been recent advances in treatment of colorectal cancer, gene expression array has the potential to improve the application of these therapies by the use of tumorspecific "fingerprints". Recently a molecular signature of 43 genes was developed predicting the outcome for clinical stages within a 90% accuracy.[78] A 23-gene signature could predict recurrence in Dukes' B patients to be upstaged to receive adjuvant therapy.[79]

Further research aims not only at finding new molecular targets but also for the pre-

diction of sensitivity to therapy. Future care may soon incorporate the data derived from a single micro-array chip that will describe a patient's tumor, predict prognosis, and direct specific therapy.

## Aims and outline of the thesis

Since Fearon and Vogelstein in 1990 presented the genetic model for the adenocarcinoma sequence of colorectal cancer, many prognostic studies varying from early stage markers to markers involved in late progression and liver metastases have followed.
As has become evident from this introduction there is an ongoing need for prognostic markers that can be used for individualized prediction of clinical outcome.

*Chapter 2.*
Many systems are available for the detection of occult tumor cells in the bone marrow, blood and lymph nodes of cancer patients. In this chapter an overview is given of the various commercially available automated microscopy systems, and their capabilities. Furthermore the current status of the application of these instruments for bone marrow, blood and lymph nodes is presented.

*Chapter 3.*
Spread to locoregional lymph nodes is one of the most important prognostic indicators of the TNM classification.
Detection of micrometastases in node-negative patients might upstage patients in need for additional chemotherapy. In this chapter an approach is described by which immunohistochemical staining and multiple sectioning is combined and is subjected to novel high-throughput automated imaging.

*Chapter 4.*
The presence of tumor cells in the bone marrow (BM) of cancer patients has shown to be related to a worse prognosis. This paper describes the use of array-CGH to detect genome alterations (gains and losses) in primary tumor tissue from BM-positive patients compared to matched (on stage and site) BM-negative patients. A higher number of differential aberrations and a distinct chromosome pattern, confirmed by interphase FISH, were found in the BM-positive group as compared to the BM-negative group.

*Chapter 5.*
While analyzing primary tumor tissue for a pilot study for array-CGH it was noticed that the set of patients with bad prognosis could not be analyzed, due to the fact that the amount of tumor material was less than 50%. This lower threshold is important for array-CGH to obtain reliable DNA profiles of the tumor cells and to avoid contamination with normal cells. Morphological evaluation of H&E stained sections showed that these tumors with bad prognosis had a high proportion of stroma and few tumor cells. The tumors with good prognosis showed the opposite, abundant tumor and less stroma. This phenomenon has led to the prognostic evaluation of this parameter in a larger patient study of which the results are shown in this chapter.

*Chapter 6.*
In this chapter the work presented in chapter 4 was continued but now focused on stage I-II colon patients. This subgroup of patients is in need for additional markers

to select specific "high risk" patients. Immunohistochemical staining of three molecular markers known to be involved in stroma production was performed. SMAD4 expression status was found to further improve the prognostic value of the presence of stroma in the primary tumor.

*Chapter 7.*
The conclusions of the studies presented in this thesis and the future perspectives of the presented parameters are discussed in this chapter.

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# Chapter 2

## Detection of tumor cells in bone marrow, peripheral blood and lymph nodes by automated imaging devices

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Review

# Detection of tumor cells in bone marrow, peripheral blood and lymph nodes by automated imaging devices [1]

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**Abstract.** The presence of tumor cells in bone marrow, peripheral blood and lymph nodes has proven its clinical and prognostic value. Since the frequency of these cells in bone marrow and blood is sometimes as low as 1 per million and due to the fact that for the analysis of lymph nodes many sectioning levels have to be analyzed, automated imaging devices have been suggested as an useful alternative to conventional manual screening of specimens. The aim of this paper is to review the performance of current equipment that is commercially available, based on literature published so far. Requirements for introducing this equipment for routine clinical practice are discussed.

Keywords: Disseminated tumor cells, micrometastases, immunohistochemistry, image analysis, automated devices (microscopy)

## 1. Introduction

Prognostication in case of newly diagnosed cancer is of pivotal importance. The presence of tumor cells outside the primary tumor is associated with a worse course of disease. Depending in which organ tumor cells are detected, different names have been coined for these cells. Generally one speaks about micrometastases when lymph nodes (LN) are considered, disseminated tumor cells (DTC) in case of bone marrow (BM) and circulating tumor cells (CTC) in case of peripheral blood (PB).

1. The search for metastases in lymph nodes (LN) involvement is an established prognostic factor for recurrence and survival [13,35,51]. The current routine method for the detection of metastases exists of surgery of the primary tumor and resection of the surrounding LN followed by pathological investigation of one routine heamatoxylin and eosin (H&E) stained section per node.

In case of epithelial tumors the application of immunohistochemistry (IHC) for cytokeratin (CK) specific antibodies can be applied to improve the ability to recognize smaller size metastasis and single disseminated epithelial cells. In combination with the analysis of multiple sections per node this results in the detection of up to 35% more positive nodes as compared to conventional histopathology [1,12,26]. This method is particularly applied for thorough examination of the sentinel lymph node (SLN) biopsy, which is the first node to which the tumor metastasizes. For breast cancer it has been shown that the SLN can predict axillary status in 95% of cases [24,46,49,53]. Further, in a significant percentage (18%) of patients the detection of micrometastases ($<$0.2 mm) is accompanied by second echelon axillary lymph node metastases [45,50].

2. The detection of disseminated tumor cells in BM is a new prognostic marker. Both in breast cancer as in colorectal cancer is the presence of tumor cells in BM a strong predictor for the development of overt

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metastases. Although recent publications convincingly show that the presence of DTC in BM is an important prognostic marker with clinical impact, it has not become routine clinical practice [10,36,40,42,47,54]. To make this step, large randomized clinical trials have to be performed. This demands a standardized protocol, for both sample processing and staining as well as for specimen analysis and interpretation of the obtained results. Such protocols have to be robust and reliable but also cost effective.

3. The presence of CTC in PB appears to be an early marker for recurrence and relapse as has been demonstrated for breast cancer patients [14,15]. However for this new prognostic marker the level of evidence has not yet reached that as for BM.

In a consensus meeting held at the 5th International Symposium of Minimal Residual Cancer in San Francisco September 2005 a uniform protocol was discussed for the processing and analysis of BM samples to detect DTC. Also the use of an automated imaging device was suggested to detect low frequency DTCs and CTCs since many cells have to be analyzed to obtain statistical reliable quantitative results. Furthermore since investigation of multiple sections increases the chance for detecting CK positive cells in LN automated imaging devices also have advantages in case of LN section analysis.

Other methods to be considered for detection of rare cells are (RT) PCR and flow cytometry. PCR, when specific markers are available, theoretically offers unparalleled sensitivity (up to 1 tumor cell in $10^7$ or better), but this method is hampered by high false positive determinations [17,19,30]. Furthermore no visual control of the detected events is possible. Flow cytometry is a powerful technique to analyze a large number of cells in a few minutes, but lacks the sensitivity needed for this purpose.

Manual screening of IHC stained slides is time consuming and is prone to human error particularly when multiple slides or sections have to be analyzed. In the past, automated microscopy using image analysis has been introduced for automated screening of large amounts of cells to detect one single occurring cell [6,41]. Proper interpretation of immunocytochemical (ICC) staining along with careful morphological assessment appears imperative to assure that (ICC) stained cells are, in fact, tumor cells, as opposed to leukocytes or other non-neoplastic cells [7].

At least two models, including variations, have been proposed for the process of metastases. According to the first model the primary tumor is biologically heterogeneous and metastatic capacity is acquired late in tumorigenesis [22]. The second model supports the hypothesis that the capacity to metastasize is acquired early during tumorigenesis and is intrinsic to the malignancy. Consequently, metastases can be found very early from the onset of disease [4]. In the last model early detection of the disseminated cells is of pivotal importance for further patient treatment. Recently Kang et al report on a variant of these theories, still only proven in mouse models, that within the primary tumor subpopulations are present with a genetic signature which codes for the site of preferential homing [29]. One of the shortcomings of these tumor models is that they insufficiently take the various pathways of dissemination into account, such as haematogenic and lymphatic dissemination [42].

Current research finds new hypothesis for tumor models and metastatic spread. According to the latest theory only the stem cells are responsible for metastasis formation [21]. Analysis of these cells is important for the understanding of the tumor biology. Automated imaging in combination with laser-capture techniques can play an important role in the detection, identification and analysis of such selected cells using molecular techniques.

In this review article we have evaluated the performance of automated imaging devices for the detection of occult tumor cells in BM, PB and LN, its present status for implementation in routine pathology diagnostic and to some attend its implications for patient management.

## 2. Automated imaging devices

Commercially available systems typically consist of an automated microscope, a personal computer and a camera, mostly of the CCD type (charged coupled device), for acquiring image data. Most functions of the microscope such as filter selection, focusing, stage movement, selection of magnification and of the dichroic filter cubes in case of fluorescence are controlled by the PC. The PC also processes the images of the detected cells. These system parameters largely determine the performance of the automated devices. The CCD camera can be a color or black/white (b/w) model. In the b/w model artificial color images can be produced by combining sequentially recorded monochromatic red, green, blue (RGB) images using the proper filter settings. Devices are available for the au-

tomatic loading of slides on the microscope stage. This means that large batches of slides can be automatically processed and analyzed overnight. The capacity of these slide loaders varies from 8 to 100 slides.

Analysis can be performed using a conventional bright-field microscope or be based on fluorescence. Bright-field microscopy has the advantage over fluorescence that the image acquisition is faster. In addition slides stained immuno-enzymatically for bright-field analysis are stable over a long period and archival material can be used. A disadvantage is that the number of antigens that can be stained simultaneously is limited; in practice not more than two. Fluorescence microscopy offers the advantage of multi-marker assessment. However acquisition of images takes more time, since sufficient numbers of photons must be integrated to provide good quality images. In some cases photobleaching offers a problem and automated focusing in fluorescence is more complex and time consuming than in case of bright-field imaging.

According to Abbe's law the spatial resolution depends on the wavelength of the light and the numerical aperture of the objective lens. Optimal results are obtained if sampling occurs according to Nyquist, that is the sampling frequency should be at least twice the optical resolution. For example, when the spatial resolution is 0.5 $\mu$m sampling should be at least 0.25 $\mu$m. In case of digital image acquisition also the pixel size of the CCD camera is relevant, as this relates directly to the sampling frequency.

Obviously trade off's between resolution and speed can be made. For most systems switching between objectives is performed to increase efficiency. Scanning is then performed first on lower magnification to select regions of interest and subsequently only these regions are analyzed at a higher magnification for the detection of positive events.

A typical analysis procedure involves hands off analysis of a preset area. During the automated analysis the slide is moved by an automated scanning stage. Generally, stepping or DC motors are used that run at speeds varying between, for instance, 20–200 $\mu$m/sec depending on the screw spindle used. This implies that in principle a slide is scanned in several seconds. However, this is the movement only and does not take into account the start/stop commands, the recording of the image by the CCD camera and the time needed for automated focus. Generally, stage movement is not the speed limiting step in automated analysis. Autofocussing can be performed on regular fields e.g. every 6th field, or be based on bilinear interpolation between predefined focus points. Cells are usually selected on the basis of intensity and color of the applied ICC staining in combination with shape and size of the positive stained cells. Artifacts such as degenerated cells, clumps of cells and debris can be recognized as such by optimized image analysis algorithms based on shape and intensity parameters. Selected cells are stored in image memories for review by the pathologist; in addition, of all selected cells corresponding coordinates $(x, y, z)$ are saved which allows for visual inspection using reallocation under the microscope and subsequent confirmation or revision of the selected tumor cells by the operator. Often an overall low magnification digital image is produced by stitching the individual recorded images together. Essential is that proper corrections are made for image overlap (typically, scanning is performed with some overlap in fields in order to prevent the missing of important events) and for imaging errors such as shading. Some commercial systems provide bright-field as well as fluorescence analysis. Typically IHC stained cells are detected on the basis of bright-field microscopy, but further analysis of selected events of multiple fluorescence markers (either FISH or ICC) is subsequently applied for confirmation or to obtain additional prognostic information [37].

Besides conventional microscopy (image plane scanners), also object plane scanners exist, often used in the fluorescence mode. Examples are laser scanning systems [11,25,28]. Krivacic et al. reported on a fiber-optic array scanning technology (FAST) which applies laser-scanning techniques in combination with automated digital microscopy offering a considerable advantage in terms of speed over conventional automated (fluorescence based) microscopy [34].

In conclusion, to assess the performance of an automated scanning system, key parameters are: (1) Speed: what is the estimated frequency of the rare event to be detected, and how many cells have therefore to be analyzed? Example: for a frequency of 1 in $10^5$, at least $10^6$ cells should be analyzed. (2) Image quality: determined by the optics [numerical aperture (NA)] and magnification of the objective and the type of CCD camera (number of pixels). Note that speed and image quality are related: a higher magnification leads to a smaller field of view and therefore to longer scanning and analysis time. Also, the higher the numerical aperture, the smaller the depth of focus, implying that autofocussing becomes more critical and may take more time. (3) Robustness: largely determined by the software, in particular in situations of unexpected events

in the sample such as large areas of dirt, air bubbles, empty fields, etc.

Other techniques for the detection of occult tumor cells include the enumeration of epithelial cells, which are separated from the blood by antibody-coated magnetic beads and identified with the use of fluorescently labelled antibodies against CK and with a fluorescent nuclear stain; a process that takes place in a specially designed cell chamber [15]. For slide based systems cells preferentially should be deposited on a fixed location of the glass slide, evenly distributed to avoid overlapping cells and preferentially as flat as possible for easy focusing. Staining of the cells of interest with specific monoclonal antibodies facilitates automated analysis since cells are selected on basis of the specific color of the immuno-enzymatic substrate. Regular staining and analysis of positive and negative control samples is essential to monitor possible variables both in staining and system performance. For most systems software has been optimized to not miss any positive cells, thereby generating an affordable number of artifacts.

### *2.1. Types of automated systems*

Two commercial systems are available based on bright-field microscopy and single marker detection for cells in BM, PB and LN; the ACIS system from ChromaVision Medical Systems, Inc (San Juan Capistrano, CA) and the ARIOL-SL50 system (formerly MDS system) from Applied Imaging Corp. (Santa Clara, CA) [2,8].

Furthermore the Metafer 3.0 (MetaSystems GmbH, Altlussheim, Germany) represents an example of an automated microscope system based on fluorescence imaging, that has been successfully used to detect small numbers of tumor cells in PB and BM [37]. Finally, there exists a system named REIS frequently reported in the literature for the detection of tumor cells in PB, which however is not commercially available [31–34]. As previously described fluorescence-based analysis allows combining multiple markers to characterize the DTC. The ARIOL and Metafer system therefore have incorporated the possibility to combine the scanning process in absorption with re-analysis of selected events in fluorescence at higher microscopic magnification.

The CellSearch System (Veridex) has a somewhat different set up than the other systems. It consists of a semi-automated system for the preparation of a sample and a semi automated fluorescence-based microscopy system but cells remain in a chamber while analyzed using the microscope [14,15]. See also Table 1.

Next to the above mentioned systems, other slide scanning systems (e.g. Aperio) are available but these lack the dedicated application for the detection of disseminated cells.

## 3. Detection of disseminated tumor cells in bone marrow

In a large meta-analysis of 4703 patients with stage I, II, or III breast cancer, the prognostic significance of the presence of DTC in the BM at the time of diagnosis was evaluated and found to be strongly associated with a poor prognosis [9]. The results of this study are of significant clinical value and would considerably impact nowadays patient management in case all breast cancer patients would be subjected to a BM aspirate to search for DTC. Therefore attempts have been made to analyze BM samples automatically.

In a recent, study Bauer et al. evaluated the use of the ACIS system for identifying DTCs in human breast cancer BM specimens in a study of normal BM, spiked BM samples and BM samples obtained from breast cancer patients [2]. Cells were stained using ICC for cytokeratin with New Fuchsin as a substrate for alkaline phosphatase and hematoxylin as a nuclear counterstain. The spiked BM samples were all found positive whereas for the 39 originally reported negative breast cancer patient samples 17 (44%) were scored positive for the presence of CK positive cells by the pathologist after automated analysis. Furthermore this study reports a $\sim$11.9-fold reduction in pathologist review time for automated microscopy using ACIS-assisted analysis, relative to manual microscopy.

### *3.1. Performance of automated analysis compared to conventional manual analysis*

In a paper by Borgen et al. the detection sensitivity of automated imaging using the MDS/ARIOL system was compared to conventional manual screening [8]. Two pathologists analyzed clinical BM slides from patients with breast cancer. Slides were stained for CK using IHC. The automated procedure detected 50 out of 52 (96%) positive samples whereas only 32 (62%) were found by pathological examination. The two missed samples contained abnormal cells that were located outside the screening area defined for the automated analysis.

Table 1

| | ACIS | ARIOL | REIS | Metafer 3.0 | CellSearch System |
|---|---|---|---|---|---|
| Detection system | Absorption | Absorption and fluorescence | Fluorescence | Fluorescence and absorption | Fluorescence |
| Microscope | Standard bright field. Type not known | Olympus BX61 | Nikon Eclipse 1000 | Axioplan 2 (Zeiss) | Cell Tracks Analyzer II |
| Camera | 3-chip Sony DX 9000 | CCD Cohu | CCD b/w Sensicam | CCD | CCD |
| Software | Own design Color detection | Own design Color, morphology and fluorescence based detection | Image-Pro Plus 4.5 | Own design | Own design. Fluorescence detection |
| Time to analyze $1 \times 10^6$ cells | 14 min | 15 min | 20 min | ** | Contents of 7.5 ml blood in 7 min |
| Time for reviewing* | 2 min/slide | 1–2 min/slide | 2 min/slide | data not given | Related tumor cell number and tumor cell debris |
| Capacity | 100 slides/run | 50 slides/run | 1 slide/run | 8 slides/run | 9 cartridges/hour |
| Magnification | 10×* | 10×* | 4×* | variable | 10× |
| Evaluation of detected cells at higher magnification | Second scan: ×40, ×60 | Second scan: ×20, ×40, ×60 | Second scan: ×10, ×20, ×40, ×60 | Second scan: ×63, ×100 | |
| Application | BM, PB, LN | BM, PB, LN | PB | BM | BM, PB |
| Target staining (enzyme or direct label) | Cytokeratin (alkaline phosphatase) | Cytokeratin (alkaline phosphatase) | Cytokeratin (rhodamine) | Cytokeratin (FITC) | EpCAM (ferrofluid) |
| Additional features | | Reanalysis of selected objects using fluorescence mode | Total cell count based on DAPI image | Total cell count based on DAPI image | Confirmation selected cells: Cytokeratin, CD45, DAPI |

*Evaluation at higher magnification with interactive artifact rejection software based on shape and size.
**According to information from brochure; acquisition up to 7000 cells/second, analysis time is unknown.

Kraeft et al. found in a study of 39 BM samples from breast cancer patients, fluorescently stained for cytokeratin, that 44% of the positive samples were missed by manual screening but positively identified using the ACIS system [2].

Becker et al. analyzed 298 BM slides from patients with breast cancer, stained for bright-field using the 2E11 monoclonal antibody. Manual analysis of 178 cytospin samples did not show any tumor cells, whereas the ACIS system was able to detect tumor cells in 43 (24%) of these cases [3].

The results of these studies show that automated imaging devices are able to detect DTC in the mononuclear cell fraction with a sensitivity that is at least equal but often better than manual screening.

The reliability of the automated screening procedure has been tested by Borgen et al. for the MDS/ARIOL system in a series of repeated measurements using slides stained for CK using IHC [8]. They found that the screening analysis was stable from day to day for a single machine and from one machine to the other. The variation of the system in the detection of the number of objects to be verified by the operator was 8–9%; the reproducibility in detecting true positive cells was 100% over a period of 5 days [2].

## 4. Detection of circulating tumor cells in peripheral blood

Since tumor cell dissemination into the BM takes place via blood circulation, it should be possible to detect these cells in a PB sample. A simple blood test would, in contrast to BM aspiration, allow for the analysis to be frequently repeated and be used for staging at diagnosis as well as for therapy monitoring and long-term patient management. Circulating tumor cells (CTC) have been successfully detected and isolated from blood but not yet with an unequivocal consensus [20,27,43,44], although recently a large technical progress in this field has been made by Veridex [14,15]. The frequency of positive cells in the different published studies varies considerably. These variations can be due to the analysis of different tumor types but can also be a result of a limited sensitivity of the available assays. The biology and clinical importance of CTC's in PB is far from understood.

Kraeft et al. used the REIS system and investigated its sensitivity for the analysis of PB samples from cancer patients [33]. Data from 80 slides obtained from breast and lung cancer revealed that as many as 14 of 35 positive slides (40%) were missed by manual screening but positively identified by REIS.

Witzig et al. examined the feasibility for identifying and enumerating CK+ cells in the PB of breast cancer patients using anti-EpCAM-conjugated immunomagnetic beads for carcinoma cell enrichment by automated detection using the ACIS system [55]. Twenty-eight percent of the breast cancer patients demonstrated CK+ cells including 76% of patients with metastastic disease, 8% with N+ disease and none of the patients with N− disease. A further study should demonstrate a possible correlation with prognosis [55]. This group performed an additional study to investigate the reproducibility of the ACIS system for tumor cell detection. Four slides were independently analyzed using 3 separate systems. All four specimens revealed identification of identical numbers (reproducibility 100%) of cells (range 1–49) for three systems [55].

Till now only two studies have analyzed the impact of finding CTC on disease free and overall survival. The main reason is that it is important to first develop and rigorously test the performance of isolation and detection of these cells before large-scale clinical studies are undertaken. Pierga et al. report a study on the comparison of blood and BM samples from 114 breast cancer patients at different stages of disease (I–IV) [43]. Cytospin Ficoll fractions of mononuclear cells were analyzed from both PB and BM, stained for the cytokeratin monoclonal antibody A45B/B3 and automatically analyzed with ACIS. CK+ cells were detected in 28 (24.5%) patients in blood and in 67 (59%) patients in bone marrow. The presence of CK+ cells in blood strongly correlated with the presence of CK+ cells in BM. Twenty-six (93%) patients with CK+ cells in blood also had positive BM ($p < 0.001$). Positive cells were detected in the peripheral blood from 7.5% of stage I/II patients, 36% in stage III and 41% patients with stage IV ($P = 0.017$). The presence of circulating CK+ cells in PB did not statistically correlate with disease-free survival. However, the authors conclude that this method of detection may be useful to monitor the efficacy of treatment in advanced metastatic disease.

Recently, Cristofanilli et al. published two papers in which the presence of CTC was evaluated in a prospective study for patients with metastatic breast cancer. Patients were also monitored for 6 months after treatment initiation. Detection of CTCs before and after initiation of first-line therapy in patients with metastatic breast cancer were found highly predictive factors for progression-free ($P = 0.0014$) and overall survival ($P = 0.0048$) [14,15].

## 5. Detection of micrometastases in lymph nodes

Strategies for investigating SLN were designed in a consensus meeting of the College of American Pathologists primarily aiming to detect metastases of size 2 mm or larger, since only the presence of metastasis of this size had been shown to convincingly correlate with survival [23]. It was furthermore recommended to apply multiple sectioning of whole SLN at 2 mm intervals in order to increase detection sensitivity.

Despite the increased workload many centers apply multiple sectioning at even smaller intervals, since it has been shown that the sensitivity for detecting occult cells and micrometastasis <0.2 mm increases up to a certain level with the number of sections investigated [50]. Although they may not have prognostic impact with respect to overall survival, such micrometastases or even single cells are in a significant percentage of patients accompanied by second echelon metastases, thereby being clinically relevant as they indicate the need for further axillary dissection [16,45,50].

IHC staining using cytokeratin specific antibodies is applied to improve the recognition rate of smaller size

metastasis and single tumor cells in particular. As an example, the application of IHC in combination with the analysis of multiple sections results in the detection of up to 35% more positive nodes as compared to conventional histopathology [1,12,26].

Basically, micrometastasis have been further defined with a lower limit and are designated pN1mi for metastases >0.2 mm but not >2 mm. The category of metastases that are <0.2 mm have been designated isolated tumor cells and are classified pN0 with modifiers to indicate method of detection as follows: pN0(I−) IHC performed but negative; pN0(I+), IHC positive but no clusters larger than 0.2 mm; pN0(mol− and +) identical for PCR" [18,48].

One of the key questions will be, if manual methods provide sufficient sensitivity and if examination of multiple sections can be performed in a cost-effective way. The rate of missed metastasis (micro or macro) in routine diagnostic pathology ranges from 2% to 9% [48]. Metastases may also be missed even when IHC staining is applied, which is ascribed to human failure and fatigue; problems that can be circumvented to a large extent when automated detection is applied.

### *5.1. Performance of automated analysis compared to conventional manual analysis*

Recent studies have indicated that automated imaging devices are potentially more sensitive than manual microscopy [38,52]. In a study by Weaver et al. 100 IHC-negative SLN biopsies were re-analyzed by automated imaging. Additional micrometastases, were detected in 10.4% of the cases that were originally negative classified as node negative using conventional manual microscopy, and revealed the presence of single tumor cells and groups up to 30 $\mu$m in size [52].

In a recent published study our group has reported results on the use of an automated image device to reanalyze sections from SLN that had been IHC stained as part of routine common practice in two clinical centers in the Netherlands [39]. Automated imaging did not misclassify one single positive node. However, in a significant percentage (5.9% and 12.5%) of nodes manually classified as negative, tumor cells and even groups of cells were detected and confirmed by the pathologist. Moreover, it was shown that due to a high degree of automation the pathologist's operational time at the instrument was kept within constraints, which enables the introduction of this new technology in routine clinical pathology.

## 6. Conclusions and future prospects

Based on studies reported so far it is justified to conclude that automated imaging devices are potentially more sensitive, reproducible, and accurate than routine pathological evaluation for rare events like disseminated tumor cells in bone marrow, peripheral blood and lymph nodes. Automated analysis is also faster due to the possibility to analyze large sets of slides overnight. Thereby it offers the possibility to analyze a larger part of the patient sample (more LN sections, more BM and PB slides from one patient), which is essential in situations where the disseminated cells are at very low frequency. Concluding one can state that (1) the methodology is ready, (2) the clinical impact is proven, (3) thus it is time for implementation. The first question that will rise is, if these systems are ready for routine use in pathology laboratories. As far as their performance is concerned, most likely yes. Whether this can be done in a cost effective way largely depends on the workload c.q. number of patients for which this type of analysis is meaningful. The problem is that most hospitals will not have enough patient material to effectively utilize the capacity that is provided by the 24 hour analysis capability of some machines. That is, to say not within the current clinical practice. However if for all major cancers, for which PB, BM and LN examinations are considered meaningful, automated analysis would be applied, then one machine for an average size hospital probably would be enough. This workload strongly depends on the embedding of above mentioned PB, BM and LN analysis in the clinic when clinicians are enthusiastic to change the current concept and request for these examinations. Clinical implementation will also depend on the fact to what extent a positive result of these assays will have therapeutic consequences. As a result the operational costs of these systems can then be reimbursed by regular health insurance.

When applying an automated system to detect CTCs, DTCs or micrometastasis in case of different tumor types, it is strongly recommended to fine tune the software algorithm for cell selections for each type of cancer on a representative set of tumor cases (learning set) and subsequently test these (test set) prior to clinical applications. Besides tumor type, the variability of staining protocols is a potential performance determining factor that should be controlled in a similar way.

The second question is whether detection (and enumeration) of IHC positive cells is enough for proper patient management. Current research focuses on the

development of new markers that further characterize these cells [5]. Such markers may provide information about their proliferative activity, their metastatic potential, or the predictive value for therapy outcome. By the improvement of the array techniques the analysis of single cells will become a realistic option. Automated imaging devices can assist in this technology. There is the feasibility to search for target cells on basis of cellular characteristics (CK, EpCAM, e.g.) and transform the coordinates of the found objects to laser microdissection systems (LMD). Once specific markers are found, this can be translated to FISH probes and reanalyze the slides by the same system for further characterization which will aid in specific and accurate analysis of these single cells. This may lead to new markers that can be used to help in therapy selection and predict outcome and as such contribute to patient management.

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# Chapter 3

## Automated analysis of multiple sections for the detection of occult cells in lymph nodes

*Clin Cancer Res 2003; 9(13):4826-4834*

# Automated Analysis of Multiple Sections for the Detection of Occult Cells in Lymph Nodes

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## ABSTRACT

***Purpose:*** **At present, reverse transcription (RT)-PCR against carcino-embryonic antigen mRNA is one of the few research tools for the detection of occult cells in histopathologically assessed negative lymph nodes from patients with colorectal cancer. The aim of this study was to investigate the suitability of supervised low-resolution image analysis of immunohistochemically stained sections as alternative.**

***Experimental Design:*** **Multiple sections ($n = 50$) of regional lymph nodes from patients with colorectal cancer were immunohistochemically stained and analyzed by applying low-resolution image analysis (flatbed scanning) for semiautomated detection of cytokeratin (CK)-positive stained cells. The sensitivity of this approach was demonstrated for 20 patients with stage II colorectal cancer and compared with RT-PCR regarding the detection of clinically assessed recurrence of disease within 10 years.**

***Results:*** **$CK^+$ cells were detected in all of the patients ($n = 6$; 100%) with recurrence, compared with five patients (83%) found positive by carcinoembryonic antigen RT-PCR. From patients ($n = 14$) who did not develop a recurrence, eight (57%) had positive lymph nodes. In all patients with recurrence, we visually identified at least one group of $CK^+$ cells (≥2 cells).**

***Conclusions:*** **Automated image analysis is a promising tool for the detection of occult cells in histopathologically negative nodes. It is potentially more sensitive but less specific for detecting recurrence of disease than conventional histopathology or RT-PCR and is particularly useful for the evaluation of sentinel nodes. Furthermore, it opens new ways for basic research of occult cells based on molecular profiling after laser-microdissection.**

## INTRODUCTION

The presence of lymph node metastasis is one of the most important prognostic factors and therapeutic selectors for many types of cancer. Despite the prognostic value of lymph node status as assessed by conventional histopathology, a significant percentage of patients with node-negative colorectal carcinoma (diagnosed as lymph node negative by conventional examination) develop recurrence of disease (1–3). Reasons for such exceptions are the biology of the tumor but also the limited sensitivity of conventional histopathology to detect rarely occurring occult cells in lymph nodes. For practical reasons, only a few H&E-stained sections are examined, which limits the sensitivity of the technique to detect occult tumor cells.

Recently, alternative approaches to detect occult cells in lymph nodes have been described. Liefers *et al*. (1) examined lymph nodes from a group of 26 patients with stage II colorectal cancer, originally reported as negative by histopathology, using RT-PCR[2] against CEA mRNA. In their retrospective study, 14 of 26 patients were reported as positive for CEA. The 5-year recurrence-free survival for the CEA-positive patients was 50% and for CEA negative patients, 91%. Others found similar results (2–3).

A different approach to increase sensitivity is by IHC. This method relies on the analysis of immunohistochemically stained sections of lymph nodes for the detection of immunostained cells. It has been reported for patients with breast carcinoma that the application of IHC in combination with the analysis of multiple sections results in the detection of up to 35% more positive nodes as compared with conventional histopathology (4–12). The clinical value however of this observation needs to be confirmed.

The sensitivity of the detection of occult cells has been shown to increase with the number of sections per lymph node examined up till a certain number of sections, (13), but the practical applicability of multiple sectioning is limited by

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[2] The abbreviations used are: RT-PCR, reverse transcription-PCR; IHC, immunohistochemistry; CEA, carcinoembryonic antigen; ADASP, Association of Directors of Anatomical and Surgical Pathology; LUMC, Leiden University Medical Center.

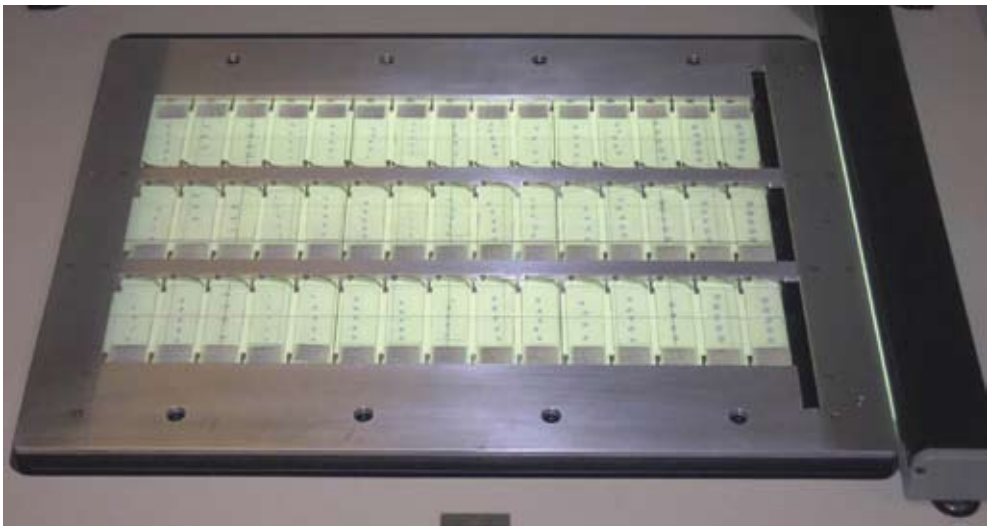

*Fig. 1* A3-size mold to hold 15 × 3 = 45 slides on the AGFA XY15 scanner. Each slide may contain several tissue sections.

the labor intensive nature of preparing a large amount of immunohistochemically stained sections. For the analysis of sentinel nodes, however, multiple sectioning is highly recommended by the ADASP and is considered practically feasible (7).

In this article, we describe an approach in which immunohistochemical staining and multiple sectioning is combined and is subjected to novel high-throughput automated imaging. This imaging system uses a high-performance flatbed scanner (FBSc), which is able to digitize, in one A3 format, hundreds of cytological or histological specimens. Digitally acquired images of the immunohistochemically stained tissue are then automatically analyzed for the presence of positive-stained occult cells. Storage of cell coordinates allows for direct morphological evaluation using conventional microscopy.

In this study, we have compared visual examination of CK-immunostained serial sections (as gold standard) with automated analysis and with the RT-PCR data from the referred Liefers article (1) with the emphasis on the sensitivity of the method to detect patients with recurrence of disease within a period of 10 years after a diagnosis of colorectal cancer tumor-node-metastasis stage II.

## MATERIALS AND METHODS

**Patients.** From 20 patients with tumor-node-metastasis stage II ($T_2N_3M_0$) colorectal cancer, lymph nodes were obtained consecutively from curative resections performed at the Department of Surgery of the LUMC between January 1990 and February 1992 (stage I cases are rare, and were not available in this department). From this material, originally studied using RT-PCR by Liefers *et al.*, 119 of 246 lymph nodes were still available (1). Twenty-one blocks showed poor material not suited for analysis (fat tissue, degenerated material, no histological material remaining).

Preoperative and perioperative examinations of the patients showed no evidence of metastatic disease. Follow-up was carried out in accordance with the department's protocol (Department of Surgery, LUMC) and was based on periodic evaluations of the patient (1). The follow-up of the patients was at least 10 years and was updated by checking the patient files as of February 1, 2002.

**Sectioning and Immunohistochemical Staining.** In the original study, one-half of the node was fixed in formalin and was embedded in paraffin for routine histopathological examination (1). The other half of the resected node was used for RNA isolation for the analysis of CEA-specific mRNA using RT-PCR.

For the present study, all of the available lymph nodes were analyzed for those patients who were originally PCR-positive for CEA. From the PCR-negative group for each patient, six lymph nodes were chosen randomly to match the average number of nodes in the positive group.

From this material, serial sections (10 sections of 5 μm in series at each level) were cut with intervals of 200 μm until no material was left in the paraffin block. Sections were stained for CK using the antibody AE1/AE3 (14).

The sections were hydrated and were subjected to sodium citrate (0.01 M, pH 6.0 at 100°C) for 10 min before incubation with a mixture of primary biotinylated monoclonal antibodies against CK AE1/AE3 (Dako, Glostrup, Denmark). Immunostaining was based on the avidin-biotin-peroxidase technique using 3,3′-diaminobenzidine (DAB) as

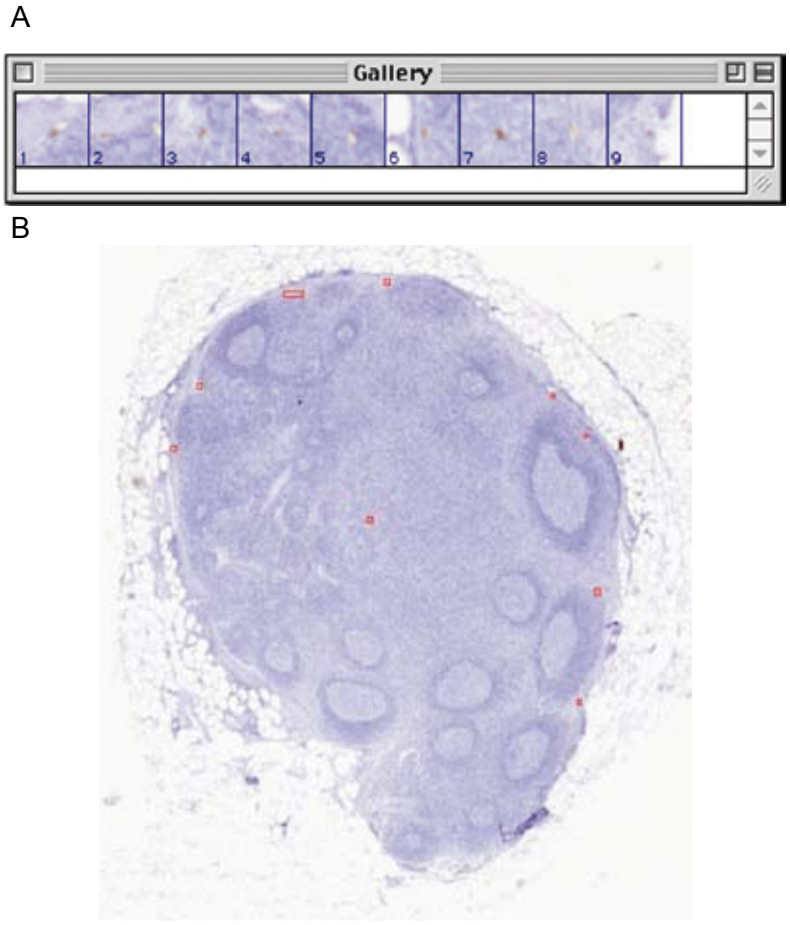


*Fig. 2* *A,* gallery with images of candidate occult cells detected in a lymph node section of a patient with colorectal carcinoma. Selection of candidate cells is based on immunoperoxidase (*brown*) staining for CK. Counterstaining is performed with hematoxylin (*blue*). *B,* overview of the lymph node with markers displaying the location of the candidate cells.

the end point product; all of the sections were counterstained with hematoxylin.

**Method of Analysis of the Slides.** All of the IHC slides were first manually examined in a very thorough way. The results served as the gold standard for the automated analysis. A node was called positive when at least one immunohistochemically positive cell was found (excluding WBCs, macrophages, *e.g.,* known for nonspecific staining) confirmed by a second person (a pathologist).

Subsequently sections were recorded using the flatbed scanner. Automated analysis was performed on all of the recorded nodes. The location of all manually detected $CK^+$ cells was marked on printouts of the recorded images and compared with the automated analysis.

**Automated Analysis.** The system consists of a flatbed Agfa XY-15 scanner interfaced to a 933MHz Power Mac G4 computer via a SCSI-2 interface. The optical resolution was 5000 dpi in both directions corresponding to a pixel distance of 5-μm image acquisition.

A special mold has been constructed to scan a maximum of 45 microscopic slides automatically (Fig. 1). Digitization was performed using the ColorExact software package from Agfa. In addition to the system software, we designed dedicated image analysis software to analyze the bed-scan for the presence of microscopic slides within the mold and the lymph node sections on each slide.

The analysis of the lymph node sections was divided into the following steps: the selection of a lymph node, the

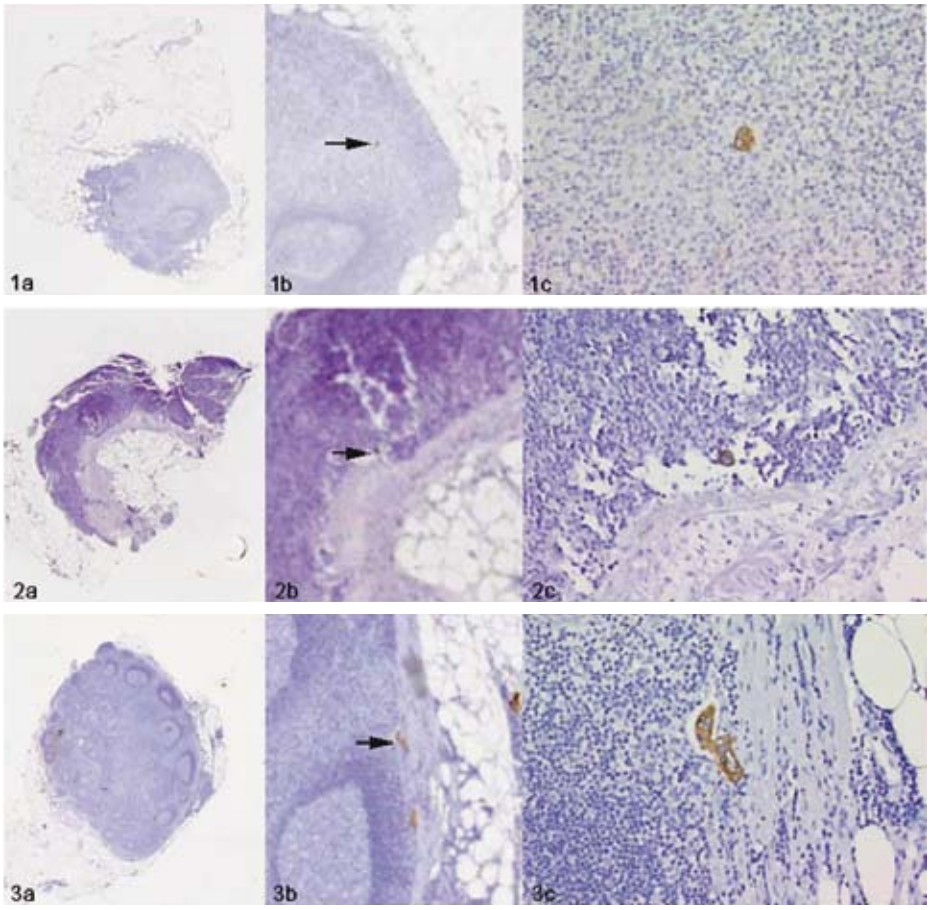


*Fig. 3* *a,* examples of images recorded by the flatbed scanner at 5600 dpi. Sections of lymph nodes from patients with colorectal carcinoma. Cells are positively stained for CK and counterstained using hematoxylin. *b,* zoom function of recorded image; *arrow,* a small group (*3b*) or single $CK^+$ cells (*1b, 2b*). *c,* conventional microscope image of the same cells. Recorded with color CCD camera using ×16 objective.

detection of candidate occult cells, and the measurement of cell features (area, shape, peak intensity, and averaged probe color). On the basis of these features, eventual falsely selected objects (*i.e.,* other than $CK^+$ cells) were recognized as such. The analysis of a lymph node section resulted in a gallery of images of the candidate occult $CK^+$ cells found and an overview image of the lymph node with position markers where the events were found (see Fig. 2). On the basis of the gallery, the majority of falsely selected objects was easily recognized visually. When needed, the stored cell coordinates were used to relocate events by automated microscopy for visual interpretation at high spatial resolution (Fig. 3).

The total time necessary to automatically process a lymph node, which involved 80 slides with 5 sections per slide, was 81 min, of which 1 min. was required to make a full bed-scan at lower resolution and to determine the coordinates of the image crops for the scanning of the individual sections.

For the optimization of the selection algorithm of the automated analysis program, a positive node was analyzed. Parameters were set on the detection of groups and single $CK^+$

*Table 1* Number of automatically detected $CK^+$ cells and groups in lymph nodes of patients with and without recurrence of disease

| Disease status | Recurrence ($n = 6$) | Nonrecurrence ($n = 14$) |
|---|---|---|
| Positive patients | 6 (100%) | 8 (57%) |
| Positive lymph nodes | 17/43 (40%) | 16/77 (21%) |
| Patients with $CK^+$ cells | 6 (100%) | 8 (57%) |
| No. of $CK^+$ cells | 81 | 40 |
| Patients with $CK^+$ groups[a] | 6 (100%) | 5 (36%) |
| No. of $CK^+$ groups[a] | 31 | 12 |

[a] "groups" is defined as $\geq 2$ $CK^+$ cells.

cells. Recorded images were automatically analyzed, and the results were compared with those obtained by conventional microscopy. Using optimized selection criteria for automated analysis, we detected 34 (94%) of 36 visually recognized cells by automated analysis. These algorithms were used for the present study.

**Statistical Analysis.** We have reanalyzed the set of patients previously investigated by Liefers *et al.* (1) because of the availability of the material and the long-term follow-up (10 years). Obtained results using our method (IHC-automated analysis) were compared with the updated data of the RT-PCR study on the same set of patients. Carefully performed manual examination of the same slides served as the gold standard for this comparison.

First, the automated analysis was compared with the manual evaluation. The data were described comparing patients with a recurrence of disease *versus* nonrecurrence, regarding IHC-automated analysis (Table 1) and RT-PCR (Table 2).

Then, IHC was compared with RT-PCR by calculating the sensitivity and specificity (Table 3). Finally, we calculated the optimal distance for the sectioning of the paraffin material to detect all of the patients with a recurrence of disease (Fig. 4).

## RESULTS

**IHC-Automated Analysis Compared with Conventional Microscopy.** A total of 119 lymph nodes (from 20 patients) were available; 33 were found positive and 65, negative, and 21 were not analyzed (see "Material and Methods"). Per lymph node on average, 49 (range, 8–81) histological sections were analyzed. Comparing automated analysis with visual analysis using conventional microscopy revealed that, from a total of 33 visually evaluated positive nodes, two nodes were missed using automated analysis. Both nodes contained only one cell resulting in a sensitivity of 94% to detect a positive *node*. However, both patients had three more nodes in which occult cells were detected. Therefore, no positive *patients* were missed by automated analysis.

One patient was missed with visual analysis but was found positive on visual verification of the candidate cells after automated analysis. This case illustrates the imperfectness of conventional screening compared with an automated performance of the image analysis procedure.

Table 4 presents the results of the lymph node analysis and the clinical outcome of the 20 patients.

*Table 2* Results of RT-PCR for CEA expression (same patients as shown in Table 4)[a]

| Disease status | Recurrence ($n = 6$) | Nonrecurrence ($n = 14$) |
|---|---|---|
| Positive patients | 5 (83%) | 3 (21%) |
| Positive lymph nodes | 12/45 (27%) | 5/109 (5%) |

[a] Data from Liefers *et al.* (1).

*Table 3* Calculation of the sensitivity and specificity of both methods (IHC-automated analysis/RT-PCR) with respect to the detection of patients with recurrence of disease

| | Sensitivity | Specificity |
|---|---|---|
| IHC-automated analysis cutoff level: 1 $CK^+$ cell | 100% | 43% |
| RT-PCR | 83% | 79% |

**IHC-Automated Analysis Compared with RT-PCR.** All of the patients ($n = 6$) who had developed a recurrence of disease were detected by IHC-automated analysis (Table 1). From 43 analyzed nodes in this patient group, 17 (40%) were found positive for CK-stained cells with on average eight cells per positive node. In all of these patients, groups of cells (number cells $\geq 2$) were visually recognized.

From all other patients ($n = 14$) who did not develop recurrence of disease, 8 (57%) were found positive. In 16 (21%) of 77 nodes analyzed in this group, $CK^+$ cells were found. Only five patients (36%) had cells located in groups. One patient (7 $CK^+$ groups) died within 1 month after surgery because of a gastric hemorrhage. One patient died from a cause other than disease, and three patients showed no evidence of disease 10 years after surgery. In three patients, no groups of cells were detected.

Using the RT-PCR method, we detected five (83%) patients with recurrence of disease. Forty-five nodes were analyzed in this group, of which 12 (27%) were found positive for CEA expression. Three patients (21%) with no recurrence of disease had positive lymph nodes (Table 2).

**Sensitivity and Specificity.** Realizing that a relatively low number of patient samples have been investigated, we, nevertheless, calculated the sensitivity and specificity of both methods.

The sensitivity of IHC-automated analysis to detect $CK^+$ nodes in patients with recurrence of disease was 100%; the specificity was 43%. The sensitivity and specificity of the RT-PCR method on the same set of patients studied by Liefers *et al.* (1) are, respectively, 83 and 79% (Table 3).

**Sampling Distance of Paraffin Blocks.** By reanalyzing the data, we calculated the effect of the distance of sectioning of the paraffin material with respect to successful detection of recurrence of disease. Fig. 4 shows the effect of varying the distance between sections with respect to the detection of positive lymph nodes ("positive" was defined here as containing *at least* one $CK^+$ cell).

Additionally, the percentage of lymph nodes is shown in

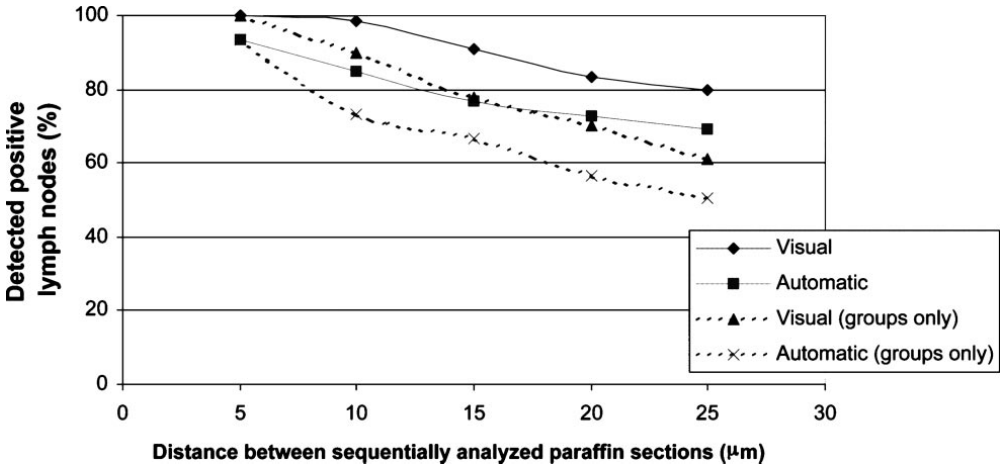


*Fig. 4* Percentage of detected lymph nodes as function of the distance between sequential sections for both visual and automated detection based either on $CK^+$ cells or on positive groups of cells only. A lymph node was classified as positive when at least one verified $CK^+$ cell (or a group, ≥2 cells) was detected.

which at least one group of cells (≥2 $CK^+$ cells) was found. The sensitivity of both the visual and automated detection decreases from 100 and 93% to, respectively, 98 and 85% when one-half of the number of sections is analyzed. One $CK^+$ lymph node containing one group (2 cells) was missed when only one in every two sections was analyzed. When the detection of at least one $CK^+$ positive group of cells was used as the criterion, sensitivity decreased from 100 to 90% and from 93 to 73% for automated and visual inspection, respectively.

## DISCUSSION

This study shows that visual examination of multiple immunohistochemically stained sections for the presence of occult tumor cells can be automated by low-resolution image analysis. As such, this method may serve as a useful alternative for RT-PCR, particularly when large numbers of sections are analyzed (at least 50 per node for this study). In all of the patients ($n = 6$) with recurrence of disease, immunohistochemically positive cells were detected using this method, demonstrating its feasibility for this application. Compared with the RT-PCR study previously performed by Liefers *et al*. (1), automated analysis of immunohistochemically stained sections appeared to be more sensitive in detecting patients with recurrence of disease (100 *versus* 83%). We also found more positive nodes in this group (40 *versus* 27%). However, in 57% of the patients who did not develop a recurrence of disease, positive cells were also found, compared with 21% for the RT-PCR method. When the cutoff level was increased from one cell to two $CK^+$ cells, the specificity increased from 43% to 57%. This makes automated analysis more sensitive but less specific than the referred RT-PCR method and, therefore, requires additional analysis of the detected positive cells.

The number of analyzed patient samples, however, is considered too small to conclude that the differences are significant and meaningful. Discrepancies with the RT-PCR results may be attributable to unavoidable sampling errors. Yasuda *et al*. (15) analyzed six serial sections using IHC and detected micrometastases in 92% of patients with recurrence but also found a high percentage of positive patients in the nonrecurrent group (70%). When more specific markers than pan-CK are available as published by Izbicki *et al*. (16) for esophageal cancer (BerEp4), the high rate of false positive findings can possibly be reduced, thereby increasing the specificity of the current assay.

It is estimated that routine H&E analysis has only a 1% chance of identifying a focus of cancer cells less than three cells in diameter (17). This level of sensitivity implies the finding that about 25% of patients with colorectal cancers who are node negative by routine H&E examination may develop distant metastases (1–4). The histopathological criteria for occult metastases are far from clear. Most of the studies identifying occult tumor cells have been performed in breast cancer. For instance, Turner *et al*. (18) and Kell *et al*. (19) report that, for patients with breast cancer with minimal axillary involvement, the presence of efferent vascular invasion or nodal hilar tissue invasion and the location of a micrometastasis in sinusoidal rather than parenchymal tissue may indicate a less favorable prognosis. Others have suggested the inclusion of the size of the metastasis, groups *versus* single cells, and the microanatomical location of occult cells as prognostic features (20). However, evaluation of the potential value of these parameters has not been systematically pursued.

*Table 4* Detection of micrometastasis and outcomes of patients with stage II colorectal cancer

| | Lymph nodes | | Outcome | |
|---|---|---|---|---|
| Patient no. | Examined | Positive | Vital status | Disease status |
| 1 | 4 | 3 | Dead | Recurrence[a] |
| 2 | 10 | 1 | Dead | Recurrence |
| 3 | 2 | 1 | Dead | Recurrence |
| 4 | 3 | 2 | Dead | Recurrence |
| 5 | 17 | 4 | Dead | Recurrence |
| 6 | 7 | 6 | Dead | Recurrence |
| 7 | 3 | 0 | Dead | Other[b] |
| 8 | 6 | 0 | Dead | Other |
| 9 | 5 | 0 | Dead | Other |
| 10 | 4 | 2 | Dead | Other |
| 11 | 6 | 3 | Dead | Other |
| 12 | 6 | 0 | Dead | Other |
| 13 | 5 | 0 | Alive | NED[c] |
| 14 | 10 | 4 | Alive | NED |
| 15 | 6 | 0 | Alive | NED |
| 16 | 4 | 1 | Alive | NED |
| 17 | 10 | 2 | Alive | NED |
| 18 | 4 | 2 | Alive | NED |
| 19 | 6 | 1 | Alive | NED |
| 20 | 1 | 0 | Alive | NED |

[a] Recurrence death from local or distant recurrent disease.
[b] Other death from a cause other than cancer.
[c] NED, no evidence of disease.

Although RT-PCR methods have been investigated to improve sensitivity, specific markers still do not exist for many tumors. RT-PCR is proven to be very sensitive and able to detect 1 in 10 million cells but is often prone to false positive classification because of contamination, and illegitimate low-level expression of marker transcripts in normal lymph nodes has also been reported (21). Furthermore, PCR has the restriction that detected events cannot be morphologically evaluated and confirmed.

Microscope-based analysis has the advantage of enabling morphological analysis of the detected occult cells by the pathologist. The use of monoclonal antibodies to further characterize the detected cells (either by bright-field or fluorescence microscopy) may be useful (22). It is evident that the number of positive nodes will increase on analysis of more sections, and may strongly increase (23). Automated analysis as described here may be further improved by connection of the scanner to an off-line automated microscope for rapid relocation of the detected events and by fine tuning of the cell classification algorithm with respect to the accuracy. The analysis is rather time consuming, and speed can be increased but is, when focusing on sentinel nodes, not a prerequisite. For use in a clinical setting, the sectioning and staining of all resected lymph nodes is too labor intensive. However, for the analysis of sentinel nodes, which most of the time involves only one to three lymph nodes, serial sectioning is highly recommended by the ADASP and is practically feasible (7).

The clinical significance of immunohistochemically detected tumor cells present in excised lymph nodes in case of colorectal cancer remains unclear. In a recent multi-institutional study of 736 patients with breast cancer, the presence of immunohistochemically detected occult cells in axillary lymph node metastases was found to be significant and, in case of postmenopausal women, was an independent predictor of overall survival. For colorectal cancer, this reaffirms the need for larger studies with longer follow-up (4).

The relatively large data set (4569 sections of 119 nodes of 20 patients) of the presented study allowed us to examine the effects of the sectioning density on the detection of $CK^+$ cells in a particular lymph node. It appeared that the number of $CK^+$ cells decreased inversely proportional to the sampling distance (data not shown). This relation suggests that the $CK^+$ cells are more or less randomly distributed throughout the node. Because the presence of $CK^+$ cells was rather low in a number of $CK^+$ lymph nodes, a large proportion of the lymph node has to be analyzed to classify the node as positive. As can be seen in Fig. 4, one $CK^+$ lymph node containing only two $CK^+$ cells (group) was missed (even visually), when only one in every two sections was analyzed. When the detection efficiency of lymph nodes with at least one $CK^+$ group is considered, one would expect that detection of groups, being larger than single cells, would be less dependent on the sectioning distance. Fig. 4 shows the opposite, however. This can be explained by the low frequency of groups present in most of the lymph nodes when compared with the number of single $CK^+$ cells and by the fact that most of those groups consisted of only 2 to 3 cells.

To better understand the biology of metastasis, research is needed to further characterize the detected cells, which may ultimately lead to an increase in specificity and diagnostic accuracy. Such information can be obtained by phys-

ical isolation of these cells by laser microdissection followed by single-cell RT-PCR and an analysis of gene composition (24).

Such research has recently been published by Klein *et al.* (25), who found a different genetic make-up for single cells *versus* groups of cells. The clinical importance of these findings, however, is not yet known.

This information can be used to produce specific markers for diagnostic assays that may ultimately allow the identification of biologically important populations of cells that can be directly linked to clinical outcome.

The present study was performed on a well-documented selection of colorectal patients and served as a model. It is obvious that a similar approach is also indicated for breast cancer or for melanomas in which lymph node involvement and, particularly, the role of the sentinel node is an important focus of research (8, 9, 26–31).

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# Chapter 4

Differences in genomic profiles of colorectal tumors of patients with and without disseminated tumor cells in the bone marrow

# Differences in genomic profiles of colorectal tumors of patients with and without disseminated tumor cells in the bone marrow

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**Abstract.** *Purpose:* The presence of disseminated tumor cells in the bone marrow (BM) of colorectal patients is correlated with worse prognosis. The goal of our study was to identify differential chromosomal aberrations for patients with and without disseminated tumor cells to identify patients at risk for tumor cell dissemination to the BM. The DNA profile of CRC tumors from BM-positive and BM-negative patients was analyzed. *Methods:* Using standard methods DNA was isolated from 34 tumors (stage I-IV), from 17 BM-positive patients and 17 BM-negative patients. Comparative genomic hybridization was performed using home printed 1 Mb genomic arrays (3500 BAC clones). Patients in the BM positive and negative group were matched for tumor-site and stage. Confirmation of aberrant copy numbers was performed using interphase fluorescence in situ hybridization (FISH). *Results:* For both BM-positive and BM-negative patients common chromosomal changes were found as generally seen for CRC. A higher number of alterations (n=318) was observed for BM-positive patients as compared to BM-negative patients (n=240). Differential analysis of both patient groups showed chromosome regions 6p (p21.1), 9p (p11.2-p13.3), 12q (q13) and 16 and 19 (both full chromosomes) most frequently gained, whereas losses were observed for chromosome 11q (q22.3-q25) and 15q (q11.2-q12 and q14-q21). These findings were confirmed by interphase FISH. A minimum number of three out of seven altered chromosomes was selected to optimally discriminate between BM-positive and BM-negative patients. These three alterations were found more frequently for BM-positive patients (59%) as for BM-negative patients (12%) (chi square $p<0.05$) and also appeared to correlate with a higher chance to develop distant metastases. Also, a small recurrent amplification for chromosome 13q12 was found in a small set (n=4) of BM positive patients harboring FLT1 (VEGFR1) a gene involved in angiogenesis. *Conclusion:* This paper describes a novel set of genomic alterations associated with the presence of disseminated tumor cells in the BM. These chromosomal areas harbor genes that could be involved in the overall metastasis process of the tumor.

## 1. Introduction

The presence of disseminated tumor cells (DTC) in the bone marrow (BM) of cancer patients has shown to be of prognostic importance. For breast cancer this parameter is well established and has shown to be a better prognostic discriminator for survival compared to lymph node status.[1, 2] For colorectal cancer patients it is known that the presence of tumor cells in BM seems to be an independent prognostic

factor but needs to be confirmed in larger patient series.[3-9]

Despite the high tumor load released in the peripheral blood and lymph nodes, only few cells survive and settle in the bone marrow, but not all cells grow out to metastases. Genomic studies suggest that the primary tumor possesses the capacity to metastasize already in a very early stage of tumorigenesis and that this prognostic signature is maintained in lymph node and distant metastasis.[10] However, most strategies for the analysis of the primary tumor fail to take the cellular heterogeneity into account. Recent studies suggest that cells, that have disseminated from the primary tumor and migrated e.g. to the bone marrow, show a genetic profile that differs from the primary tumor. A suggested hypothesis is that these cells either undergo genetic alterations during tumorigenesis or are derived from small sub clones of the primary tumor[11,12] and could provide an explanation for the observation that metastases can still occur even years after removal of the primary tumor.[13,14]

Breast cancer patients with micrometastases in the lymph nodes (deposits of tumor cells >0.2 mm and ≤ 2 mm) are targeted for adjuvant therapy. It is known that there is a lack of effect of adjuvant chemotherapy on the elimination of single dormant tumor cells in bone marrow.[15] Molecular analysis might provide additional information to help develop therapies.[16] Ongoing research in this field aims to determine the aggressive potential of these cells and at the identification of relevant markers.[17] Questions to be addressed are: Are the phenotypic properties of these single cancer cells identical to the primary tumor?[16] Do they contribute to clinically detectable secondary metastases?[14] How do they settle and survive in their target organs?

The best way to give answer to these questions is the analysis of single cells selected from the BM. Especially the group of Klein et al had success in the analysis of single breast cancer cells, scraped from glass slides, using CGH.[16] However one should take into account the possible heterogeneity of the tumor which means that at least 10 cells from every patient should be analyzed, which is a valuable and labor-intensive method. It would be of preference if this information is already present in the primary tumor.

Similar as for breast cancer, research for colorectal cancer is focusing on the molecular biology. Genomic copy number changes are found frequently and are believed to contribute to the development and progression through inactivation of tumor suppressor genes and amplification of oncogenes. Rather the accumulation of aberrations than the sequence determines aggressiveness of the tumor.[18] Comparative genomic hybridization (CGH) was developed to allow for genome-wide screening of copy number changes.[19] Gain of chromosome 20q is a widespread finding in primary CRCs (67%) as is loss of 18q (49%).[20] Other consistent regions of copy number gain are 7p, 8q, 13q and 12p along with deletions of 8p and 4p. This is a first study comparing tumor tissue from patients with and without tumor cells present in the bone marrow to find genetic aberrations that correlate with or contribute to the dissemination to the bone marrow and give more insight into the tumor biology.

Array-based CGH results are reported

Table 1

| Patient characteristics | | | |
|---|---|---|---|
| | **Total series** | **BM positive** | **BM negative** |
| **Gender** | N (%) | N (%) | N (%) |
| **Male** | 21 (61.8) | 11 (52.4) | 10 (47.6) |
| **Female** | 13 (38.2) | 10 (76.9) | 3 (23.1) |
| **Age (yrs)** | | | |
| **< 50** | 7 (21.0) | 5 (71.0) | 2 (29.0) |
| **50-70** | 10 (29.0) | 3 (30.0) | 7 (70.0) |
| **> 70** | 17 (50.0) | 9 (53.0) | 8 (47.0) |
| **Location tumor** | | | |
| **left** | 14 (41.0) | 7 (50.0) | 7 (50.0) |
| **right** | 6 (18.0) | 3 (50.0) | 3 (50.0) |
| **rectum** | 14 (41.0) | 7 (50.0) | 7 (50.0) |
| **T status** | | | |
| **T1** | 0 (0) | 0 (0) | 0 (0) |
| **T2** | 2 (5.9) | 1 (50.0) | 1 (50.0) |
| **T3** | 31 (88.3) | 15 (48.4) | 16 (51.6) |
| **T4** | 1 (5.6) | 1 (100) | 0 (0) |
| **N status** | | | |
| **N0** | 17 (50) | 9 (52.9) | 8 (47.1) |
| **N1** | 12 (35.0) | 6 (50.0) | 6 (50.0) |
| **N2** | 5 (14.7) | 2 (40.0) | 3 (60.0) |
| **Stage** | | | |
| **I** | 2 (5.9) | 1 (50) | 1 (50) |
| **IIA** | 12 (35.3) | 6 (50) | 6 (50) |
| **IIB** | 0 (0) | 0 (0) | 0 (0) |
| **IIIA - C** | 10 (29.4) | 5 (50) | 5 (50) |
| **IV** | 10 (29.4) | 5 (50) | 5 (50) |
| **MSI** | | | |
| **MSS** | 16 (47.1) | 8 (50) | 8 (50) |
| **MSI-H** | 2 (5.8) | 0 (0) | 2 (100) |
| **Unknown*** | 16 (47.1) | 9 (56.3) | 7 (43.7) |

* Rectal cancer patients have a low change on MSI positivity, therefore staining for MSI was not applied.

from a set of 34 phenotypically well characterized colorectal cancers. This set of patients was composed on the basis of the presence of DTC's in the bone marrow. Patients with positive BM were matched with, for tumor site and TNM stage, likewise BM negative patients.

## 2. Material and methods

### Patient materials

Patients were selected from an ongoing study for the detection of disseminated tumor cells in BM within the Department of Surgery from the Leiden University Medical Center in Leiden, The Netherlands. From the data archive of this study, patients were selected with known BM status and the availability of frozen primary tumor tissue.

The series consists of 17 BM-positive colorectal carcinoma patients (selected for BM-positivity according to guidelines ISHAGE)[21] and 17 BM-negative patients all matched for tumor-site (right-sided, left-sided and rectum) and TNM classification.

From 26 patients the BM was collected before operation on the primary tumor, 8 patients were known with metastases confined to the liver and BM was collected before resection or perfusion. Four patients with rectum carcinoma received preoperative radiation therapy of which one patient also chemotherapy.

For 26 BM patients frozen tissue from the primary tumor was available. From 8 patients operated on for liver metastases, frozen tissue from the liver metastasis was used.

Patients provided signed informed consent and the study was approved by the Medical Ethical Committee of the LUMC, Leiden. All samples were handled in a coded fashion, according to National ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies).

### Bone marrow aspiration

Ten to 30 ml of bone marrow was aspirated from the anterior iliac crest prior to surgery under general anesthesia. Prior to inserting the needle in the anterior iliac crest, an incision was made into the overlying skin to prevent contamination with skin epithelial cells. Mononuclear cells were isolated from bone marrow using Ficoll gradient centrifugation and cytospinslides were prepared for subsequent immunocytochemical staining.

### Slide preparation and staining

Mononuclear cells were washed twice with phosphate buffered saline (PBS), and diluted in PBS to a concentration of 0.5 x $10^6$ cells per ml. Four ml of this suspension was evenly spread onto Histobond® adhesion microscopic slides (Marienfeld, Lauda-Königshofen, Germany) by cytocentrifugation using home made buckets.[22]

Cytocentrifugation was performed at 190g for 10 minutes in a swing-out rotor, with a controlled start and brake (Hettich, Tuttlingen, Germany) resulting in 2 million cells per glass slide. Slides were dried overnight at 37°C and stored at –70°C. The slides were stained with primary antibodies A45-B/B3 (diluted 1:100), directed against cytokeratins 8, 18 and 19 (Micromet AG, Munich, Germany) to detect DTC's or with isotype control antibodies directed against an irrelevant antigen, MOPC21, (a clone of a myeloma cell line) as a negative control staining (diluted 1:200) (BD Pharmingen, Erembodegem, Belgium). Subsequently slides were incubated with rabbit anti-mouse (1:400, DAKO) and APAAP complex (1:100, DAKO) followed by incubation with Vector-Red labeled alkaline phosphatase substrate (Brunswig). A detailed protocol has been published by Pantel et al.[23, 24] This staining resulted in a red precipi-

tate in the cytoplasm of cytokeratin-positive cells. The slides were counterstained with hematoxylin (Mayer's Hemalum; Merck, Darmstadt, Germany) to visualize nuclear morphology. The stained slides were analyzed using the ARIOL SL-50 automated microscope® (Applied Imaging a Genetix Company, San Jose, CA). One slide stained for cytokeratin and one negative control (MOPC21) slide was analyzed per patient. The features of the ARIOL system have been previously published.[25]

Criteria for evaluation of immuno-stained cells in BM were adapted from Borgen et al based on the results of the European ISHAGE Working Group for standardization of tumor cell detection. The main criteria were a large cell size, a high nuclei-cytoplasm ratio and the absence of obvious haematopoietic cell morphology.[21]

**Array-CGH**

From frozen primary tumor tissue, DNA was extracted as described by the QIAamp DNA Mini Kit by Qiagen (Westburg b.v., The Netherlands). The DNA was fluorescently labeled and compared to normal reference DNA by array-CGH using home printed 1 Mb genomic arrays (3500 BAC clones in triplicate), which were made available by the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk). The clones were grown, amplified and spotted as described previously.[26] Genomic DNA of the patient was isolated using standard techniques, and 500 ng was labeled with Cy3-dCTP (GE Health-care, Diegem, Belgium) using the BioPrimes DNA Labeling System (Invitrogen, Breda, The Netherlands). As reference DNA, 500 ng female human genomic DNA (Promega, Leiden, The Netherlands) was labeled using Cy5-dCTP. Hybridization and slide washing was performed without pre-hybridization on a HS400 hybridization station (Tecan, Giessen, the Netherlands). The arrays were scanned with a GenePix 4100A scanner (Axon Instruments, Union City, CA, USA) and the images were processed using GenePix Pro 4.1 software. Final analysis of the intensity ratios of the hybridized DNA was preformed using Microsoft Excel according to published standards[27] and VAMP software (Visualization and Analysis of array-CGH, transcriptome and other Molecular Profiles).[28]

**FISH**

Interphase FISH (fluorescence in situ hybridization) was performed on tissue imprints, according to standard protocols, to confirm full chromosome copy number changes of chromosomes 16 and 19.[29] Chromosome 10 was used as an internal (normal) control. Centromeric probes were used for chromosome 10 and 16 and three overlapping BAC clones for chromosome 19.

Scoring of the FISH signals was performed manually using a Leica DM5500 B microscope using a 400x magnification (10x ocular, 40x oil immersion N.A.1.3 objective). Four patients were selected for the evaluation of the chromosomes 10 and 16 or 10 and 19. Of each patient FISH dots in 100 intact nuclei were scored.

**Statistics**

Disease Free Survival (DFS) was defined, according to proposed guidelines, as the time from the date of primary surgery until the date of death or to the date of first loco-regional or distant recurrence (irrespective of site) or the date of a second primary tumor whatever occurs first.[30] If no recur-

rence or second primary tumor occurred DFS was calculated as the time period until date of last follow-up.
Tumor status, lymph node status and status of present metastases were applied according to AJCC/TNM guidelines.[31]
Right sided tumors were defined as: coecum, colon ascendens, flexura heaptica, colon transversum and for left sided: flexura lienalis, colon descendens, colon sigmoideum and rectosigmoideum.
Analysis of the survival curves was performed using Kaplan-Meier Survival Analysis and differences in equality of survival distributions were tested with the Log Rank Statistics. The Cox proportional hazards model was used to determine the Relative Risk (RR) or Hazard Ratio (HZ) of explanatory variables on DFS.
Using VAMP software, the suboption FrAGL (Frequency of Amplicon, Gain and Loss) displays informative regions at the probe level. For each probe, the fraction of tumors with gains and losses over the dataset was computed and displayed in the FrAGL view.[28] By performing a subtractive type of analysis from the BM-negative group versus the BM-positive group, frequently altered differential chromosome regions can be identified.

## 3. Results

### Patient demographics

A series of BM-positive (n=17) patients were matched for a control group of BM-negative (n=17) patients. The study consisted of 21 men (61.8%) and 13 women (38.2%). In 20.5% of the cases patients were younger than 50 years, 32.4% was between 50-70 years and 44.1% was over 70 years old.
In total 17 pairs of patients were available, from 13 pairs the primary tumor was analyzed; in 1 patient pair the primary tumor was located left sided, 8 pairs had a tumor right sided, 4 pairs had a rectum carcinoma.
Four patient pairs were known with metastases confined to the liver; 2 left sided and 2 rectum (from these last four patient pairs the liver tissue was analyzed as no frozen tissue of the primary tumor was available).
Eight patients developed liver metastases during follow up. For detailed TNM patient characteristics (see Table 1).

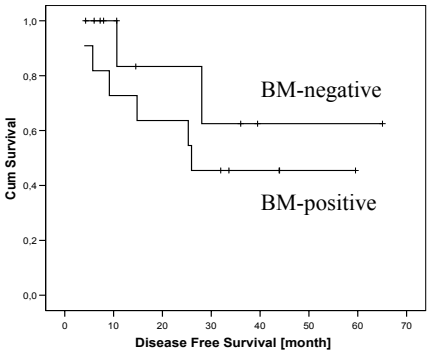
### BM status

Kaplan-Meier disease-free survival curves from BM-positive versus BM-negative patients are shown in Figure 1. The difference in survival time observed for the BM-positive group versus the BM-negative group was not found significant (p=0.3). This is most likely due to the small sample size (11 patients for each group), since the hazard ratio was found to be 2.2 (95% CI: 0.45-11.21).

### Chromosomal aberrances with array-CGH

For BM-positive patients a higher number of breakpoints estimated by VAMP was observed as compared to BM-negative patients. For the BM-positive patients group 318 breakpoints were found; 190 gains and 128 losses. For the BM-negative group this number was 240; 132 gains and 108 losses. BM- positive patients showed 31% more gains and 16% more losses than patients with a BM-negative status.
These data were correlated with the results of the meta-analysis study of Diep et al, based on CGH results of in total

Figure 1
Kaplan-Meier disease free survival curves from BM-positive versus BM-negative patients.

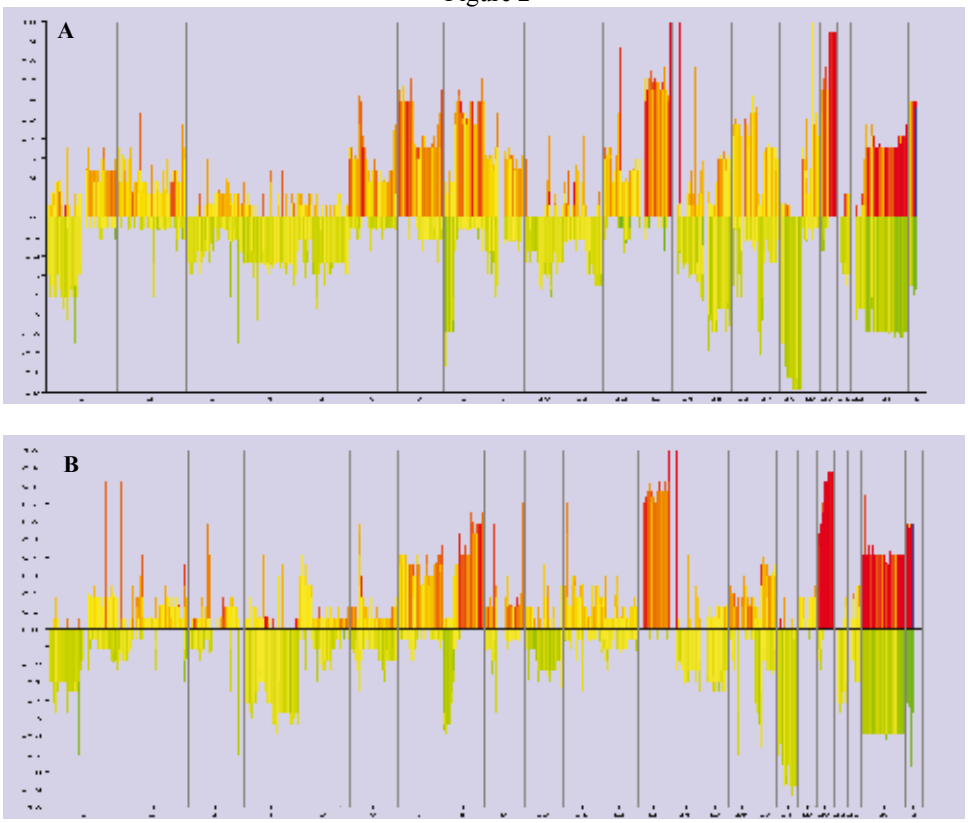



Excluded are patients from which the liver tissue was analyzed (due to lack of primary tumor tissue) and patients with synchronous liver metastasis (end point).
P value =0.3, HZ 2.2 (95% CI 0.45-11.21).

31 published studies and 859 patients.[32] In our analyzed samples we found similar chromosomal changes in the establishment of CRC as reported by Diep; early changes as gains for 8q, 13q and 20 and losses of 17p and 18, but also loss of 4p as associated with the transition from Dukes' A to B-D, deletion of 8p and gain of 7p correlating with the transition from primary tumor to liver metastasis, and late events such as gains for 1q, 12p, and 19 and losses of 14q. Figure 2.
Comparing the alterations found for the BM-positive group with the BM-negative group, differential gains for chromosome 6p (p21.1), 9p (p11.2-p13.3), 12q (q13), 16 and 19 (both full chromosomes) were most frequently found, and losses were observed for chromosome 11q (q22.3-q25) and 15q (q11.2-q12)(q14-q21).
Chromosome 6p and 15q were most frequently altered in 9 (52.9%) BM-positive patients compared to 3 (17.6%) BM-negative patients. See also Table 2 and Figures 2 and 3.
Four patients within the BM-positive group were found with an amplification within the chromosome 13q12 region, a small sized recurrent amplification with the size of ±7.9 Mb (rp11-570F6 to rp11-218E6). None of the BM-negative patients showed this amplification.
No specific recurring regions of amplifications or homozygous losses were observed for the other chromosomes.

**Genes involved in tumorigenesis**
A number of interesting genes were found within the differential gained areas of the involved chromosomes. See also Table 3.
For chromosome 6, a gain for p21.1 was found, a region including genes for: *VEGFA*, a growth factor active in angiogenesis and *CCND3,* a gene which is found upregulated in liver metastastic lesions and involved in the regulation of cyclin D3, a prominent positive cell cycle regulator.[33]
For chromosome 11, the q22.3-q25 region in which *LOH11CR2a,* a tumor-suppressor gene is located was found lost.[34]
For chromosome 12, q13 occurred in more than the average number of copies and contained genes such as: *LETMD1* also called HCCR-1 oncoprotein. This protein is reported to be involved in the tumorigenesis of breast and cervical cancer and is shown to function as a negative regulator of P53.[35, 36]
Furthermore the q13 region contains the *ACVRL1* gene, serving as receptor for TGF-B and activates the SMAD transcrip-

Figure 2




Overall frequency of DNA copy number alterations by array-based CGH. Frequency analysis (y-axis) measured as a fraction of cases gained or lost over the 3500 BAC clones.
Data are presented ordered by chromosomal map position of the clones (x-axis).
Lower bars represent losses or deletions for all clones, and the upper bars represent gains or amplifications. Red: amplification, green: deletion, yellow: below the threshold of 25%.
Data have been generated by VAMP software using the FrAGL (Frequency of Amplicon, Gain and Loss) option. a. BM-positive patients, b. BM-negative patients.

Table 2
Differential chromosomal aberrations for the BM-positive and BM-negative group, based on the array-CGH profiles.

| Chromosome gains / losses | BM-positive N | BM-positive % | BM-negative N | BM-negative % |
|---|---|---|---|---|
| **Gain 6** p21.1 | 9 | 52.9 | 3 | 17.6 |
| **Gain 9** p11.2-p13.3 | 5 | 29.4 | 2 | 11.8 |
| **Loss 11q** q22.3-q25 | 5 | 29.4 | 0 | 0 |
| **Gain 12q** q13 | 6 | 35.3 | 2 | 11.8 |
| **Loss 15q** q11.2-q12, q14-q21 | 9 | 52.9 | 3 | 17.6 |
| **Gain 16** full chrom. | 7 | 41.2 | 3 | 17.6 |
| **Gain 19** full chrom. | 6 | 35.3 | 2 | 11.8 |
| **Chromosome amplification** | | | | |
| **13** q12 | 4 | 23.5 | 0 | 0 |

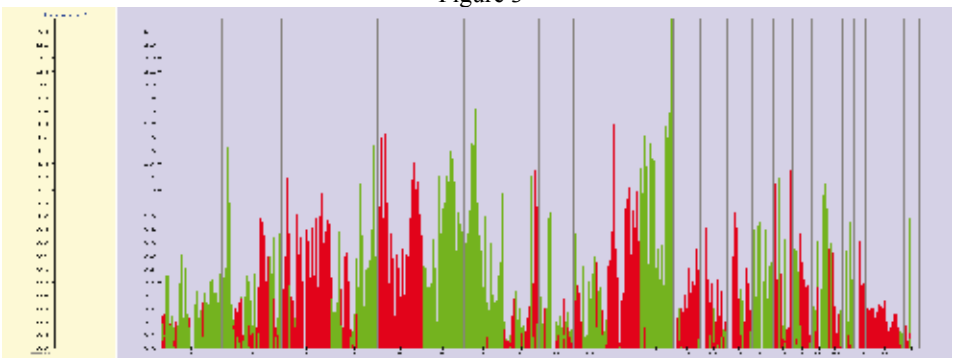
tional regulators, and the *ERBB3* (Her3) gene a member of the epidermal growth factor family (EGFR). Mutations affecting EGFR expression or activity are thought to contribute to the development of cancer.[37]

For chromosome 15, in the regions q11.2-q12 and q14-q21 less than average numbers of copies were found. *RAD51*, located in the region q14-q21, is a double stranded DNA break repair protein and involved amongst others in breast cancer. It forms a complex with BRCA1/BRCA2.[38]
For chromosome 13 an amplification for q12 was observed where the oncogene Flt1= *VEGFR1* is located*;* vascular endothelial growth factor (VEGF) is a principal regulator of vasculogenesis and angiogenesis.

**Confirmation using FISH**
Observed full chromosome copy number changes on chromosomes 16 and 19 as shown by array-CGH were confirmed by two color interphase FISH on touch print specimens. The number of FISH dots of 100 intact nuclei, hybridized simultaneously with a centromeric probe for chromosome 10 as internal control were scored. See Table 4.
In all cases, gain of the chromosomes 16 and 19 was confirmed by FISH.

**Genomic alterations in BM positive patients**
We found 7 chromosomes to be differentially altered in BM-positive patients compared to BM-negative patients. A gain for chromosome 16 and loss of 15q were most frequently seen; n=9 (52.9%) for the BM-positive patients and n=3 (17.6%) for BM-negative patients.
A minimum number of three out of seven involved aberrant chromosomes (6p, 9p, 12q, 16, 19, 11q, 15q) was chosen to optimally separate BM-positive and BM-negative patients. Using these three aberrations 10 out of 17 (59%) BM-positive patients could be identified as high-risk for the presence of disseminated tumor cells in the BM compared to 2 out of 17 (12%) of the BM-negative patients (chi-square $p<0.05$). One BM-negative patient was known with a stage-IIIc sigmoid tumor and a liver metastasis and died within 1.5 years. Notably this patient had a high-risk profile. The other BM-negative patient was diagnosed with a stage-IIIb tumor rectosigmoid and received chemotherapy. For simplicity

Figure 3



Differential analysis of DNA copy number alterations by array-based CGH of BM-positive patients compared to BM-negative patients. Frequency analysis (y-axis) measured as a fraction of cases gained or lost over the 3500 BAC clones.
Data are presented ordered by chromosomal map position of the clones (x-axis). Red: amplification, green: deletion.
Data have been generated by VAMP software using the FrAGL (Frequency of Amplicon, Gain and Loss) option.

reasons we call this "the high-risk profile". This profile was determined to most optimally distinguish between the BM-positive and BM-negative group. See Figure 4.

**Prognostic value of the high-risk profile for stage I and II patients**

In this analyzed series 14 patients (7 BM-positive, 7 BM-negative) were classified as stage I or II. In total five (71.4%) patients were identified as high-risk for the presence of DTC's using the minimum value of three out of seven involved aberrant chromosomes. Surprisingly all 5 patients were BM-positive. Three out of these 5 (60%) high-risk patients developed distant metastases during follow-up. None of the BM-negative patients showed the high-risk profile and no patients developed distant metastases.

These results show that BM-positive patients, with the high-risk profile for three involved aberrant chromosomes, have a higher change to develop distant metastases. This finding supports studies in literature where patients with positive BM have a worse outcome of disease.

**Prognostic value of the high-risk profile for patients with distant metastases**

In this series 9 out of a total of 26 patients (of which primary tumor material was analyzed) could be identified as high-risk for the presence of DTC's using the minimum value of three out of seven involved aberrant chromosomes.

Seven out of 13 (53.8%) BM-positive patients showed the high-risk profile of which 5 (71.4%) patients developed distant metastases during follow-up (additionally one patient developed a distant metastasis but without showing the high-risk profile). In contrast to 2 out of 13 (15.4%) BM-negative patients with the high-risk profile and only 1 patient having distant metastases.

Table 3
Regions and involved candidate genes of the differential chromosomal aberrations as found by array-CGH of the BM-positive versus BM-negative patient group.

| Chromosome | Distal gain | Proximal gain | Minimal size Mb | Candidate genes |
|---|---|---|---|---|
| **6 gain** | rp11-162o6 | rp11-227e22 | 3.21 | **CCND3**, **VEGFA**, CDC5l |
| **9 gain** | rp11-195f9 | rp11-113o24 | | * |
| **11 loss** | rp11-563p16 | subtelomere | 31.7 | PDGFD, CASP12, -4, -5, -1, CD3 -E,-D,-G, CBL, **LOH11CR2a**, HEPACAM, CHEK1, PRDM10, JAM3 |
| **12 gain** | rp11-571m6 | rp5-1057i20 | 10.7 | LARP4, **LETMD1**, FAM130A1, **ACVRL1,** MMP19, CDK2, **ERBB3** CDK4 |
| **15 loss** | rp11-2f9 | rp11-322n14 | 6.09 | NDN |
| **15 loss** | rp11-83j16 | rp11-154j22 | 13.58 | BMF, **RAD51**, SHF |
| **13 amplified** | rp11-570f6 | rp11-218E6 | 7.9 | **VEGFR1** |

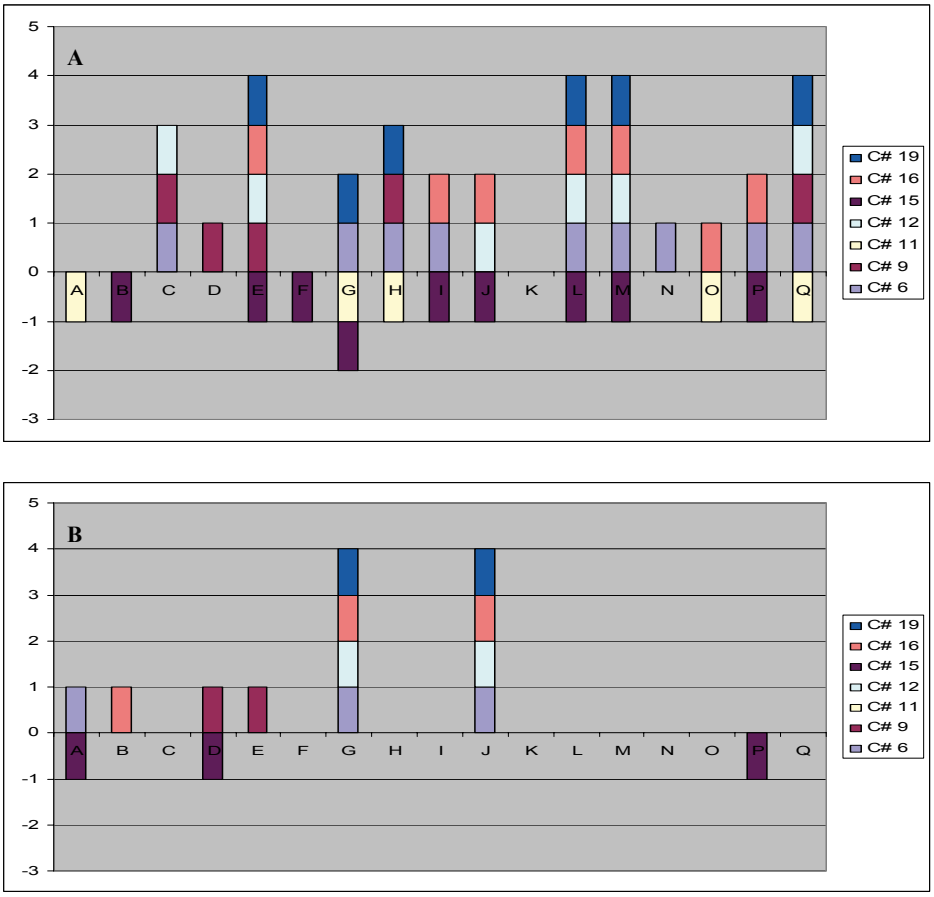
* a polymorphic region with yet not known function in tumors.

Table 4
Validation of array-CGH results for full chromosome gains of chromosomes 16 and 19 by interphase FISH. Chromosome 10 was used as internal reference.

| | **FISH*** | | | | **Array-CGH** | |
|---|---|---|---|---|---|---|
| | **C#10** | | **C#16** | | **C#10** | **C#16** |
| | mean | median | mean | median | | |
| **Patient 1** | 3.2 | 4 | 3.7 | 4 | normal | gain |
| **Patient 2** | 2.0 | 2 | 3.4 | 4 | normal | gain |
| **Patient 3** | 2.5 | 2 | 3.4 | 4 | normal | gain |
| **Patient 4** | 3.3 | 4 | 3.9 | 4 | normal | gain |
| | **C#10** | | **C#19** | | **C#10** | **C#19** |
| | mean | median | mean | median | | |
| **Patient 1** | 2.3 | 2 | 2.8 | 3 | normal | gain |
| **Patient 2** | 1.8 | 2 | 1.9 | 2 | normal | gain (small) |
| **Patient 3** | 3.1 | 3 | 3.7 | 4 | normal | gain |

* Per patient interphase FISH dots were counted in 100 nuclei.

Figure 4




DNA copy number alterations by array-based CGH of BM-positive patients compared to BM-negative patients estimated by differential analysis of FrAGL supported by VAMP software for the most frequent gained chromosomes 6, 9, 12, 16 and 19 and losses of 11q and 15q.
(X-axis: patients are displayed as numbers A-Q, Y-axis: number of alterations)
a. BM-positive patients (analyzed liver samples have the numbers: B, J, M, P), b. BM-negative patients (analyzed liver samples have the numbers: D, K, L, O).

## 4. Discussion

For this study we have applied the patients BM status information to identify genetic regions by array-CGH that might correlate with tumor cell dissemination in colorectal cancer.

As a general genome "signature" for colorectal cancer we observed the same kind of alterations as found by conventional CGH in a meta-analysis of 31 studies and 859 patients by Diep et al.[32] We also observed early chromosomal changes for CRC as gains for 8q (59%), 13q (71%) and 20 (84%) and losses of 17p (50%) and 18 (76%) and late events as gains for 1q (15%), 7p (53%), 12p (32%), and 19 (24%) and losses of 4p (24%), 8p (50%) and 14q (18%). Nakao et al analyzed 125 primary colorectal cancers using array-CGH and could identify small genomic regions on chromosome 8 and 20.[39]
Douglas et al found copy number changes, including gain of chromosomes 20, 13, and 8q and smaller regions of amplification such as chromosome 17q11.2-q12 and chromosome 4q34-q35.[40]
Within our set of patients most of the reported chromosomal changes were also frequently observed but no association could be found with the presence of DTCs. Reported gains for chromosome 11 and 17q were not frequently seen in our limited series. Gain of chromosome 17q is correlated with the transition from primary tumor to liver metastases and gain of chromosome 11 can be found by established liver metastases.[40] On contrary we observed a frequent loss of 11q in the majority of cases. Only 2 BM-positive patients and 2 BM-negative patients showed a gain for chromosome 11 of which one in each group was known with liver metastases.
For chromosome 17, three BM-positive patients and 3 BM-negative patients showed a gain of which respectively 3 and 2 were known with liver metastases. We found chromosomes 12p and 19 frequently altered in patients with liver metastases (n=6 out of 9) as described by Diep et al. to be known as late event changes in the colorectal carcinogenesis.

By using the FrAGL (Frequency of Amplicon, Gain and Loss) option as a part of VAMP software, subtraction of the BM-positive group and the BM-negative group was able to identify 7 chromosomes more frequently altered in the BM-positive group. See also Table 2. This offered a novel parameter to select for patients with tumor cells in the bone marrow which might have a higher change on the development of distant metastases.

Differential analysis of the BM-positive and BM-negative group resulted in the detection of a recurrent amplification for the BM-positive group (n=4) of chromosome 13q12 with the size of 7.9 Mb. None of the BM-negative patients showed this amplification. Within this amplified region CDK8, CDX2 and Flt1 (= VEGFR1) genes were found. Vascular endothelial growth factor (VEGF) is a principal regulator of vasculogenesis and angiogenesis.
Furthermore we found chromosome 6 (p21.1) upregulated for 9 BM-positive patients versus 3 BM-negative patients, in which the gene for *VEGFA* is located, a growth factor active in angiogenesis. Also CCND3, within the same genomic area was found up-regulated in liver metastastic lesions and is involved with cyclin D3

which is a member of the cyclin D family responsible for regulation of the initial $G_1$ to S transition.[33]

Till now, no information is published about the genetic make-up of primary tumors from patients known with disseminated tumor cells in the BM. In this pilot series, differential aberrations between the BM-positive and BM-negative group were observed, including an interesting set of frequently altered chromosomes, which also correlates with the presence of distant metastases. It should be stressed that the number of patients analyzed in this study is much too small to draw firm conclusions. This also explains why statistical analysis about the predictive value was not performed. Nevertheless a trend is observed, that should be validated and confirmed in a much larger set of well matched BM-positive and BM-negative patients.

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# Chapter 5

## The carcinoma–stromal ratio of colon carcinoma is an independent factor for survival compared to lymph node status and tumor stage

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# The carcinoma–stromal ratio of colon carcinoma is an independent factor for survival compared to lymph node status and tumor stage

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**Abstract.** *Background:* Tumor staging insufficiently discriminates between colon cancer patients with poor and better prognosis. We have evaluated, for the primary tumor, if the carcinoma-percentage (CP), as a derivative from the carcinoma-stromal ratio, can be applied as a candidate marker to identify patients for adjuvant therapy. *Methods:* In a retrospective study of 63 patients with colon cancer (stage I–III, 1990–2001) the carcinoma-percentage of the primary tumor was estimated on routine H&E stained histological sections. Additionally these findings were validated in a second independent study of 59 patients (stage I–III, 1980–1992). (None of the patients had received preoperative chemo- or radiation therapy nor adjuvant chemotherapy.) *Results:* Of 122 analyzed patients 33 (27.0%) had a low CP and 89 (73.0%) a high CP. The analysis of mean survival revealed: overall-survival (OS) 2.13 years, disease-free- survival (DFS) 1.51 years for CP-low and OS 7.36 years, DFS 6.89 years for CP-high. Five-year survival rates for CP-low versus CP-high were respectively for OS: 15.2% and 73.0% and for DFS: 12.1% and 67.4%. High levels of significance were found (OS $p < 0.0001$, DFS $p < 0.0001$) with hazard ratio's of 3.73 and 4.18. In a multivariate Cox regression analysis, CP remained an independent variable when adjusted for either stage or for tumor status and lymph-node status (OS $p < 0.001$, OS $p < 0.001$). *Conclusions:* The carcinoma-percentage in primary colon cancer is a factor to discriminate between patients with a poor and a better outcome of disease. This parameter is already available upon routine histological investigation and can, in addition to the TNM classification, be a candidate marker to further stratify into more individual risk groups.

Keywords: Colon cancer, TNM classification, primary tumor, stroma, prognosis

## 1. Introduction

Colorectal cancer (CRC) is the fourth most common form of cancer occurring worldwide, with an estimated 1.02 million new cases diagnosed each year. It affects men and women almost equally. Large differences exist in survival, associated with disease stage. It is estimated that 529,000 deaths from colorectal cancer occur worldwide annually, causing colorectal cancer to be the second most common cause of death from cancer in men in the European Union and the United States [1].

The current method for staging of colorectal cancer is according to the TNM classification. TNM is the most widely used system for classifying the anatomic extent of cancer spread and important for decision making in therapy [2]. Information on nodal involvement is an important part of CRC staging since metastasis to regional lymph nodes (LNs) is one of the most important factors relating to the prognosis of colorectal carcinomas. Patients with metastatic LNs have a shorter survival and require adjuvant systemic chemotherapy. Despite this, nodal involvement alone is not considered sensitive enough to discriminate be-

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tween patients with poor and better prognosis, because up to 20–40% of patients with invasive tumors, but without demonstrated nodal involvement, die of their cancer [3].

The five year survival for colon cancer stage II patients (AJCC staging) is 85% for stage IIA and 72% for stage IIB [4]. There is controversy in the necessity of adjuvant treatment as is shown in several studies [5–9]. During the ASCO Annual Meeting (June 2–6, 2006, Atlanta, GA) recommendations for treatment of stage II disease were proposed. Experts in GI cancer reported the results of a meta-analysis on 7 randomized trials (3,732 patients) and concluded that there is no rational to routinely apply adjuvant therapy, with the exception of high risk cases based on clinical features (T4, obstruction or perforation), nodal sampling (number of LNs resected) and prognostic factors. For some prognostic factors data exist supporting the role to select patients at risk: loss of chromosome 18q, DCC (deleted in colorectal cancer-gene) expression, DNA mismatch repair status (MMR), microsatellite instability (MSI), p53 and k-ras mutations, high thymidylate synthese (TS) expression, and circulating tumor cells in bone marrow and blood.

Currently extensive research is performed to distinguish patients with low/high risk profiles on basis of molecular techniques. Methods aiming at genomic or expression analysis using array technology or proteomics predominantly focus on the analysis of the primary tumor [10,11]. So far, genomic and expression profiling has not led to a clear set of prognostic factors that can be used for individual patient management.

Recent models on metastatic invasion focus on the tumor-"host" interface, in particular the role of the stromal tissue. The biological meaning of the stromal compartments are thought to be part of the process of wound healing in cancer, but there is also strong emphasis that CAFs (cancer-associated fibroblasts) are important promotors for tumor growth and progression [12,13].

Assuming these models are correct we anticipate that changes in the proportion of stromal compartment in the primary tumor probably reflect progression. We therefore have determined the carcinoma percentage (CP), as a derivative from the carcinoma-stromal proportion, and tested this parameter for survival. Surprisingly in a set of patients with a good and bad survival a clear difference in CP for both groups was observed. This finding stimulated us to extend our patient group for further analysis with respect to CP.

In a study of 63 colon patients (stage I–III, 1990–2001, neither pre-operative chemo- or radiation therapy nor adjuvant chemotherapy) with a mean follow up of 9.03 (SD 3.1) years we have estimated the CP on, for diagnostics used, H&E stained sections of the primary tumors and investigated its relation to overall (OS) and disease free (DFS) survival. The results of this study were then validated in a second independent study of 59 colon patients (stage I, III. 1980–1992, neither pre-operative chemo- or radiation therapy nor adjuvant chemotherapy) with a mean follow up of 16.1 (SD 4.3) years. Since for both studies OS and DFS did not differ (OS $p = 0.96$, DFS $p = 0.53$) they were also analyzed as one series.

## 2. Material and methods

### *2.1. Patient recruitment*

We selected 63 unspecified colon cancer patients with stage I–III tumors (clinically staged according to the tumor-node-metastasis classification of the AJCC [2]), who underwent curative surgery at the Leiden University Medical Center between 1990 and 2001.

For the validation study an additional 59 patients with colon cancer stage I–III were selected who also underwent curative surgery at the Leiden University Medical Center between 1980 and 1992.

None of the patients had received preoperative chemo- or radiation therapy nor adjuvant chemotherapy. Unlike the situation in the US where patients are being treated with adjuvant therapy more common, our patients were not adjuvantly treated. There were no patients included in this study with known distant metastases at surgery. Further, patients with double tumors, other malignancies in the past and death or recurrence (distant or loco-regional) within 1 month, were excluded. HNPCC patients were also excluded.

All samples were handled in a coded fashion, according to National ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies). For detailed patient characteristics see Table 1.

### *2.2. Histopathological protocol*

Pathological examination entailed routine microscopic analysis of 5 μm H&E stained sections from the most invasive part of the primary tumor. The carcinoma percentage was visually estimated by two persons (HM, WM) on the whole tumor area, on basis

Table 1
Patient characteristics

| Characteristics | Original series | | Validation series | |
|---|---|---|---|---|
| | CP-low | CP-high | CP-low | CP-high |
| Gender | N (%) | N (%) | N (%) | N (%) |
| Male | 9 (50.0) | 25 (55.6) | 10 (66.7) | 28 (62.2) |
| Female | 9 (50.0) | 20 (44.4) | 5 (33.3) | 16 (36.4) |
| Mean age (yrs)[*,**] | 69.6 (sd 15.3) | 67.6 (sd 12.3) | 65.3 (sd 12.6) | 66.8 (sd 12.5) |
| Location tumor | | | | |
| Left | 6 (33.3) | 14 (31.1) | 10 (62.5) | 18 (41.9) |
| Right | 12 (66.7) | 31 (68.9) | 6 (37.5) | 25 (58.1) |
| T status | | | | |
| T1 | 0 (0) | 4 (8.9) | 0 (0) | 0 (0) |
| T2 | 2 (11.1) | 6 (13.3) | 0 (0) | 24 (54.5) |
| T3 | 12 (66.7) | 27 (60.0) | 10 (66.7) | 20 (45.5) |
| T4 | 4 (22.2) | 8 (17.8) | 5 (33.3) | 0 (0) |
| N status | | | | |
| N0 | 4 (22.2) | 38 (84.4) | 2 (13.3) | 22 (50.0) |
| N1 | 7 (38.9) | 6 (13.3) | 9 (60.0) | 18 (40.9) |
| N2 | 7 (38.9) | 1 (2.2) | 4 (26.7) | 4 (9.1) |
| Stage | | | | |
| I | 2 (11.1) | 8 (17.8) | 0 (0) | 16 (36.4) |
| IIA | 2 (11.1) | 24 (53.3) | 2 (13.3) | 6 (13.6) |
| IIB | 0 (0) | 6 (13.3) | 0 (0) | 0 (0) |
| IIIA–C | 13 (72.2) | 7 (15.6) | 13 (86.7) | 22 (50.0) |
| Unknown | 1 (5.6) | 0 (0) | 0 (0) | 0 (0) |
| Grading (differentiation) | | | | |
| Well | 5 (27.8) | 9 (20.0) | 1 (6.7) | 9 (20.5) |
| Moderate | 10 (55.6) | 27 (60.0) | 6 (40.0) | 22 (50.0) |
| Poor | 2 (11.1) | 4 (8.9) | 8 (53.3) | 10 (22.7) |
| Unknown | 1 (5.6) | 5 (11.1) | 0 (0) | 3 (6.8) |
| MSI | | | | |
| MSS | 16 (88.9) | 34 (75.6) | 15 (100) | 34 (77.3) |
| MSI-H left sided | 0 (0) | 0 (0) | 0 (0) | 1 (2.3) |
| MSI-H right sided | 2 (11.1) | 11 (24.4) | 0 (0) | 9 (20.4) |

* Original series: mean age defined as period from birth until diagnosis. Validation series: mean age defined as period from birth until resection.
** Difference statistically not significant in the original and validation series.
All tumors were radically resected (R0).

of morphological information (for clarity reasons we only give carcinoma percentages but complementary will give the stromal percentage; e.g. CP 70% implies a stromal percentage of 30%). In case of tumor heterogeneity, areas with the lowest CP were considered decisive as is performed in routine pathology to determine tumor differentiation. Percentages were scored ranging from 20 to 90%. Percentages of 10 and 100% were not seen. Shortly the protocol: H&E sections of the tumor with the most invasive part of the primary tumor were chosen. Using a 2.5× or a 5× objective the invasive area with the desmoplastic stroma was selected. Subsequently, using a 10× objective only the fields were scored where the stroma was infiltrated with small tumor nests within all sides of the image field. The tumorpercentage was estimated (per tenfold: 10, 20, 30% etc.) per image-field. The lowest scored percentage was considered decisive. In some cases of necrosis or mucus forming tumors, scoring of the stroma percentage was more difficult and sometimes caused over- or underscoring.

For the identification of MSI-H (MSI-high) patients, 5 μm slides were immunohistochemically stained for the markers MLH1 and PMS2 [14,15].

### 2.3. Statistics

Overall Survival (OS) was defined as the time period between the date of primary surgery and the date of death from any cause or the date of last follow-up. Disease Free Survival (DFS) was defined as the time from the date of primary surgery until the date of death or to the date of first loco-regional or distant recurrence (irrespective of site) or the date of a second primary tumor whatever occurs first. If no recurrence or second primary tumor occurred DFS was calculated as the time period until date of last follow-up. To calculate Disease Specific (Overall) Survival (DS-OS) and Disease Specific (Disease Free) Survival (DS-DFS) death was restricted to death due to colon cancer.

Tumor status, lymph node status and status of present metastases were applied according to AJCC/ TNM guidelines.

Right sided tumors were defined as follows: coecum, colon ascendens, flexura hepatica, colon transversum and for left sided: flexura lienalis, colon descendens, colon sigmoideum, rectosigmoideum.

Carcinoma percentage (CP) was defined as CP-low: $<$50% including the values 20, 30 and 40% tumor and CP-high: $\geqslant$50% including the values 50, 60, 70, 80 and 90%.

Analysis of the survival curves was performed using Kaplan–Meier Survival Analysis and differences in equality of survival distributions were tested with the Log Rank Statistics. The Cox proportional hazards model was used to determine the Relative Risk (RR) or Hazard Ratio (HZ) of explanatory variables on OS and DFS.

Differences in OS and DFS between in the original series of 63 patients (original series) and the validation series of 59 patients (validation series) were tested by Kaplan–Meier Survival Analysis.

## 3. Results

### 3.1. Patient demographics

The original study (training set) consisted of 34 men (54%) and 29 women (46%), with a mean age of 68.2 years (SD 13.1; range 21.7–91.4 years). From sixty-three primary tumors 20 (32%) were located left sided and 43 (68%) right sided.

For the validation study 38 men (64.4%) and 21 women (35.6%) were included with a mean age of 66.4 (SD 12.5; range 30.1–85.0 years). Twenty-eight (47.5%) were located left sided and 31 (52.5%) right sided.

Right sided tumors included were: coecum ($n = 36$), colon ascendens ($n = 17$), flexura hepatica ($n = 8$) and colon transversum ($n = 13$) and for left sided: flexura lienalis ($n = 2$), colon descendens ($n = 1$), colon sigmoideum ($n = 32$) and rectosigmoideum ($n = 13$). For all patients tumors were radically resected (R0). For detailed TNM patient characteristics see Table 1.

### 3.2. Determination of the cut-off level for carcinoma percentage

We determined the optimal threshold level of CP on the basis of a maximum discriminating power for OS and DFS in the original study (training set) (see Table 2). This approach resulted in a cut-off point for CP at the 50% level for further analysis. Consecutively we applied this cut-off level for the validation series and the combined series. Results of the last two series were in line with those obtained for the original study.

### 3.3. Histopathology

Routine H&E stained slides from the most invasive part of the tumor were microscopically analyzed for the presence of stromal involvement using a 5$\times$ and a 10$\times$ objective. This desmoplastic stroma was not related to the total tumor size. We observed areas with

Table 2
Determination of the CP 50% cut-off value of the original series

| Carcinoma percentage | Original series | |
|---|---|---|
| | OS | DFS |
| $<$40 | 2.77 | 1.53 |
| $\geqslant$40 | 4.46 | 3.86 |
| | $p = 0.236$ | $p = 0.085$ |
| $<$50 | 1.40 | 1.36 |
| $\geqslant$50 | 5.40 | 4.82 |
| | $p = 0.001$ | $p = 0.0000$ |
| $<$60 | 3.27 | 2.26 |
| $\geqslant$60 | 5.51 | 5.31 |
| | $p = 0.016$ | $p = 0.001$ |
| $<$70 | 3.66 | 2.76 |
| $\geqslant$70 | 5.73 | 5.55 |
| | $p = 0.047$ | $p = 0.006$ |
| $<$80 | 2.85 | 3.08 |
| $\geqslant$80 | 3.14 | 5.26 |
| | $p = 0.235$ | $p = 0.058$ |

abundant stroma (CP-low) with a size as large as one microscopic field (100× total magnification), but also larger areas matching 2–4 fields were seen or even more, independent from the size of the tumor.

In general the CP was estimated on one single representative section from the primary tumor only. From 38 patients our archive contained multiple H&E stained slides from different areas of the same primary tumor, which allowed us to investigate how the scored CP percentage depended on the sampling. We noticed some heterogeneity in the CP percentage throughout the tumor. However, areas with the highest infiltration depth (T stage) had the lowest CP percentage whereas at the borders of the tumor, in case heterogeneity was found, the CP was higher. For clinical use of the CP percentage we therefore recommend the evaluation of sections taken from areas of the primary tumor with the highest T stage, which is common clinical practice.

Preliminary information of a new study by our group (to be published) shows a high agreement in the scoring for CP-low versus CP-high between three pathologists ($p < 0.0001$). Within the 27 discrepancies found for the three observers, 6 (22%) were within the 40–50% decision range.

Examples of images of H&E stained slides from the primary tumor from patients with a low CP (30%) and a high CP (80%) are given in Fig. 1. Incidentally, slides from the same tissue were differentially stained for tu-

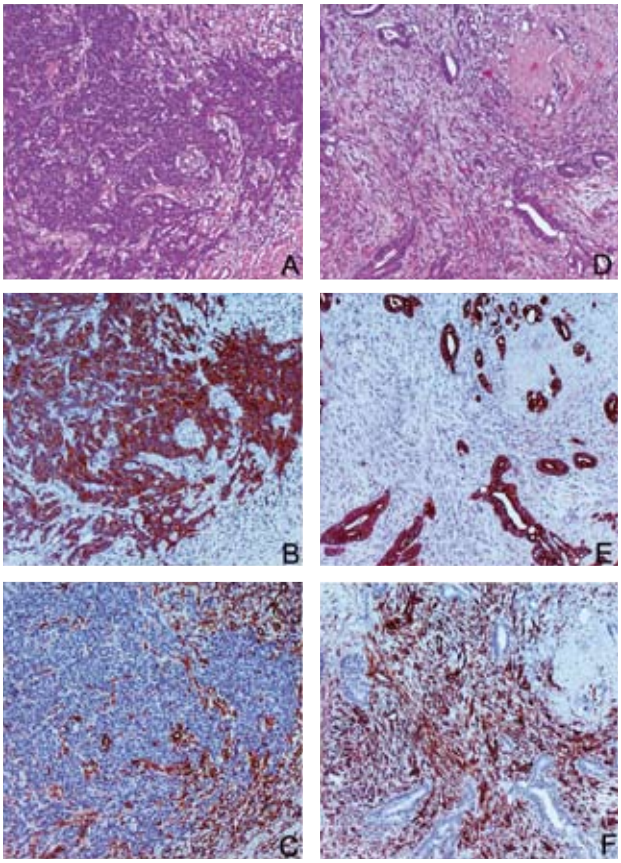



Fig. 1. H&E stained 5 µm paraffin sections of primary colon tumors. Carcinoma percentage estimated as 80% in a patient with long OS/DFS: (a) H&E staining; (b) cytokeratin staining for carcinoma cells; (c) vimentin staining of stromal compartments. Carcinoma percentage estimated as more than 30% in patient with short OS/DFS: (d) H&E staining; (e) cytokeratin staining for carcinoma cells; (f) vimentin staining of stromal compartment.

mor cells and stromal cells using antibodies specific for cytokeratin and vimentin respectively in order to check the status of the carcinoma-stromal proportion. This immunohistochemical method proved that the morphological judgment of the CP as used here was adequate.

### *3.4. Correlation with prognosis*

#### *3.4.1. Original series*

From 63 patients analyzed 18 (28.6%) had a low CP and 45 (71.4%) a high CP. The mean OS for patients with CP-low was 1.40 years and 5.40 years for CP-high ($p < 0.0001$, HZ 4.31) (DFS $p < 0.0001$, HZ 4.53). Five year survival rates for OS and DFS for CP-low compared to CP-high patients were respectively 16.7%/11.1% and 77.8%/68.9%.

CP was compared to LN status, tumor status and stage. Significant differences of OS and DFS were found, respectively for LN status and staging. Tumor status did not show significant difference. For detailed data see Tables 2, 3, 4, 5 and Fig. 2.

#### *3.4.2. Validation series*

From 59 patients analyzed 15 (25.4%) had a low CP and 44 (74.6%) a high CP. The mean OS for patients with CP-low was 1.82 years and 8.64 years for CP-high ($p = 0.0001$, HZ 3.45) (DFS $p < 0.0001$, HZ 3.91). Five year survival rates for OS and DFS for CP-low compared to CP-high patients were respectively 13.3%/13.3% and 68.2%/65.9%.

With respect to the TNM parameters significant differences of OS and DFS were found, respectively for LN status and for tumor status, but not for stage. For detailed data see Tables 2, 3, 4, 5 and Fig. 3.

Both series (original and validation) were selected on basis of the same selection criteria. Since there was no significant difference between both series for OS and DFS (OS $p = 0.96$, DFS $p = 0.52$) it was decided to combine the two sets and analyze them as one series.

In this combined series of 122 patients the OS for patients with CP-low was 2.13 years and 7.36 years for CP-high ($p < 0.0001$, HZ 3.74) (DFS $p < 0.0001$, HZ 4.18). See Tables 2, 3, 5 and Fig. 4a, b.

Table 3
P values (univariate) for CP and TNM parameters

| | Combined series | | | Original series | | | Validation series | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total $n = 122$ | Left $n = 48$ | Right $n = 74$ | Total $n = 63$ | Left $n = 20$ | Right $n = 43$ | Total $n = 59$ | Left $n = 28$ | Right $n = 31$ |
| CP | | | | | | | | | |
| OS | <0.0001 | 0.0764 | <0.0001 | <0.0001 | 0.2512 | <0.0001 | 0.0001 | 0.1594 | 0.0001 |
| DFS | <0.0001 | 0.0095 | <0.0001 | <0.0001 | 0.0811 | <0.0001 | <0.0001 | 0.0598 | <0.0001 |
| DSS/OS** | | 0.0061 | | | 0.3157* | | | 0.0056 | |
| DSS/DFS | | 0.0015 | | | 0.1942* | | | 0.0038 | |
| LN status | | | | | | | | | |
| OS | <0.0001 | 0.2446 | <0.0001 | <0.0001 | 0.0855 | 0.0002 | 0.0477 | 0.5223 | 0.0034 |
| DFS | <0.0001 | 0.1857 | <0.0001 | <0.0001 | 0.0072 | 0.0002 | 0.0347 | 0.5207 | 0.0034 |
| DSS/OS | | 0.0035 | | | 0.0472 | | | 0.0435 | |
| DSS/DFS | | 0.0015 | | | 0.0042 | | | 0.0402 | |
| Tumor status | | | | | | | | | |
| OS | 0.0091 | 0.2245 | 0.1905 | 0.1865 | 0.2458 | 0.1073 | 0.0003 | 0.0511 | 0.0071 |
| DFS | 0.0060 | 0.0378 | 0.0297 | 0.1405 | 0.0260 | 0.1881 | 0.0007 | 0.0475 | 0.0167 |
| DSS/OS | | 0.0054 | | | 0.0675 | | | 0.0031 | |
| DSS/DFS | | 0.0003 | | | 0.0104 | | | 0.0018 | |
| Stage | | | | | | | | | |
| OS | <0.0001 | 0.1905 | 0.0001 | 0.0001 | 0.1198 | 0.0006 | 0.0836 | 0.5427 | 0.0204 |
| DFS | <0.0001 | 0.0297 | <0.0001 | <0.0001 | 0.0015 | 0.0010 | 0.0518 | 0.2761 | 0.0206 |
| DSS/OS | | 0.0028 | | | 0.0510 | | | 0.0540 | |
| DSS/DFS | | 0.0001 | | | 0.0018 | | | 0.0169 | |

* Discrepancy caused by one patient outlier; low CP, long survival.
** DSS: Disease specific survival.

Table 4

Percentage of patients alive 5 years after operation for overall and disease free survival

| | Combined series | | | Original series | | | Validation series | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total | Left | Right | Total | Left | Right | Total | Left | Right |
| CP | | | | | | | | | |
| Low | 15.2/12.1* | 28.6/21.4 | 5.3/5.3 | 16.7/11.1 | 33.3/16.7 | 8.3/8.3 | 13.3/13.3 | 25.0/25.0 | 0/0 |
| High | 73.0/67.4 | 64.7/55.9 | 78.2/74.5 | 77.8/68.9 | 71.4/57.1 | 80.6/74.2 | 68.2/65.9 | 60.0/55.0 | 75.0/75.0 |
| LN status | | | | | | | | | |
| N0 | 78.8/71.2 | 70.8/58.3 | 83.3/78.6 | 78.6/69.0 | 71.4/57.1 | 82.1/75.0 | 79.2/75.0 | 70.0/60.0 | 85.7/85.7 |
| N1 | 40.0/37.5 | 42.1/36.8 | 38.1/3801 | 30.8/23.1 | 40.0/20.0 | 25.0/25.0 | 44.4/44.4 | 42.9/42.9 | 46.2/46.2 |
| N2 | 12.5/12.5 | 20.0/20.0 | 9.1/9.1 | 12.5/12.5 | 0/0 | 14.3/14.3 | 12.5/12.5 | 25.0/25.0 | 0/0 |
| Tumor status | | | | | | | | | |
| T1 | 75.0/75.0 | 50.0/50.0 | 100/100 | 75.0/75.0 | 50.0/50.0 | 100/100 | ** | ** | ** |
| T2 | 81.3/78.1 | 78.9/73.7 | 84.6/84.6 | 87.5/87.5 | 83.3/83.3 | 100/100 | 79.2/75.0 | 76.9/69.2 | 81.8/81.8 |
| T3 | 52.2/44.9 | 39.1/26.1 | 58.7/54.3 | 59.0/46.2 | 45.5/18.2 | 64.3/57.1 | 43.3/43.3 | 33.3/33.3 | 50.0/50.0 |
| T4 | 29.4/29.4 | 25.0/25.0 | 30.8/30.8 | 41.7/41.7 | 100/100 | 36.4/36.4 | 0/0 | 0/0 | 0/0 |
| Stage | | | | | | | | | |
| I | 84.6/80.8 | 75.0/68.8 | 100/100 | 80.0/80.0 | 71.4/71.4 | 100/100 | 87.5/81.3 | 77.8/66.7 | 100/100 |
| IIA | 76.6/64.7 | 57.1/28.6 | 81.5/74.1 | 80.8/65.4 | 66.7/33.3 | 85.0/75.0 | 62.5/62.5 | 0/0 | 71.4/71.4 |
| IIB | 66.7/66.7 | 100/100 | 60.0/60.0 | 66.7/66.7 | 100/100 | 60.0/60.0 | ** | ** | ** |
| IIIA–C | 32.7/30.9 | 37.5/33.3 | 29.0/29.0 | 25.0/20.0 | 33.3/17.7 | 21.4/21.4 | 37.1/37.1 | 38.9/38.9 | 35.3/35.3 |

* OS/DFS.

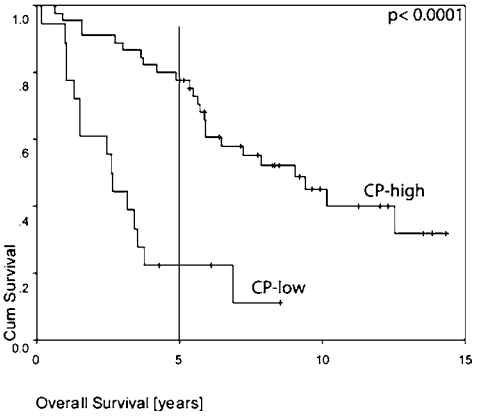
** no patients with this classification in series.

Note: for 5 year and 10 year survival comparative data were observed.

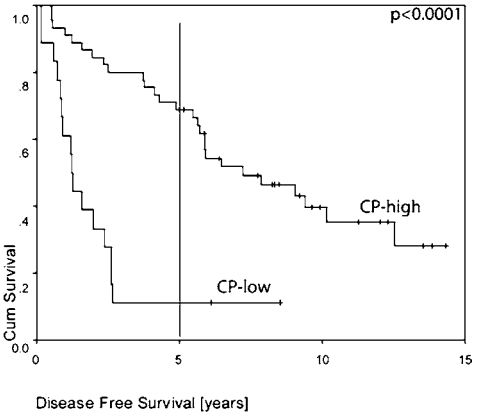
Table 5

Cox proportional Hazards regression (univariate)

| | $n$ | Topography | OS or DFS | Hazard ratio | 95% CI |
|---|---|---|---|---|---|
| Combined series | 122 | Total colon | OS | 3.74 | 2.32–6.01 |
| | | | DFS | 4.18 | 2.63–6.65 |
| | 48 | Left sided | OS | 1.98 | 0.92–4.27 |
| | | | DFS | 2.51 | 1.22–5.17 |
| | 74 | Right sided | OS | 9.56 | 4.70–19.48 |
| | | | DFS | 9.14 | 4.55–18.38 |
| Original series | 63 | Total colon | OS | 4.31 | 2.15–8.66 |
| | | | DFS | 4.53 | 2.31–8.90 |
| | 20 | Left sided | OS | 2.07 | 0.58–7.40 |
| | | | DFS | 2.75 | 0.84–8.95 |
| | 43 | Right sided | OS | 7.50 | 3.09–18.22 |
| | | | DFS | 6.15 | 2.62–14.44 |
| Validation series | 59 | Total colon | OS | 3.45 | 1.77–6.74 |
| | | | DFS | 3.91 | 2.03–7.51 |
| | 28 | Left sided | OS | 1.99 | 0.75–5.27 |
| | | | DFS | 2.38 | 0.94–6.03 |
| | 31 | Right sided | OS | 16.93 | 4.60–62.27 |
| | | | DFS | 21.06 | 5.03–88.14 |

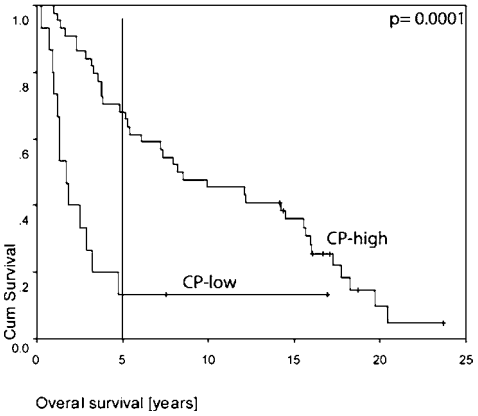



(a)

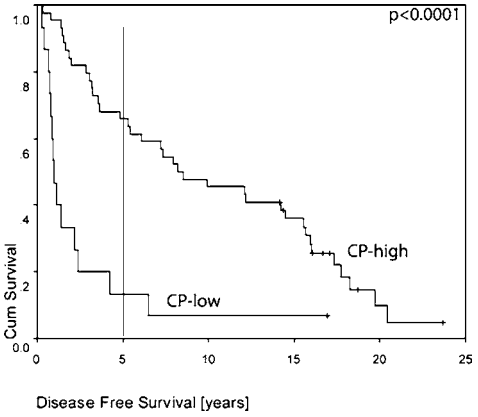



(b)

Fig. 2. Kaplan–Meier curves of the *original series* for CP-low and CP-high patients: (a) OS and (b) DFS. The dashed line indicates the 5-year survival time.




(a)




(b)

Fig. 3. Kaplan–Meier curves of the *validation series* for CP-low and CP-high patients: (a) OS and (b) DFS. The dashed line indicates the 5-year survival time.

In a multivariate Cox regression analysis, CP remained an independent variable when corrected for either stage (OS $p < 0.001$, HZ 0.39, 95% CI 0.22–0.71) (DFS $p < 0.0001$, HZ 0.34, 95% CI 0.19–0.60) or for tumor status and LN status (OS $p < 0.001$, HZ 0.37, 95% CI 0.20–0.68) (DFS $p < 0.0001$, HZ 0.34, 95% CI 0.19–0.61).

A large difference was observed between 5 year survival rates for both CP groups. A comparison with the conventional TNM parameters is given in Table 4.

### *3.5. Topography and the MSI status*

We have investigated the topography (left and right sided) and the MSI status separately, known to be parameters that have impact on prognosis.

#### *3.5.1. Left sided and right sided tumors*

The combined series consists of 122 patients of which in 39% ($n = 48$) of the cases the tumor was located left sided (a) in the colon and in 61% ($n = 74$) right sided (b).

(a) Sixteen (33.3%) of the left sided tumors had a low CP and 32 (66.7%) a high CP (OS $p = 0.0764$, HZ

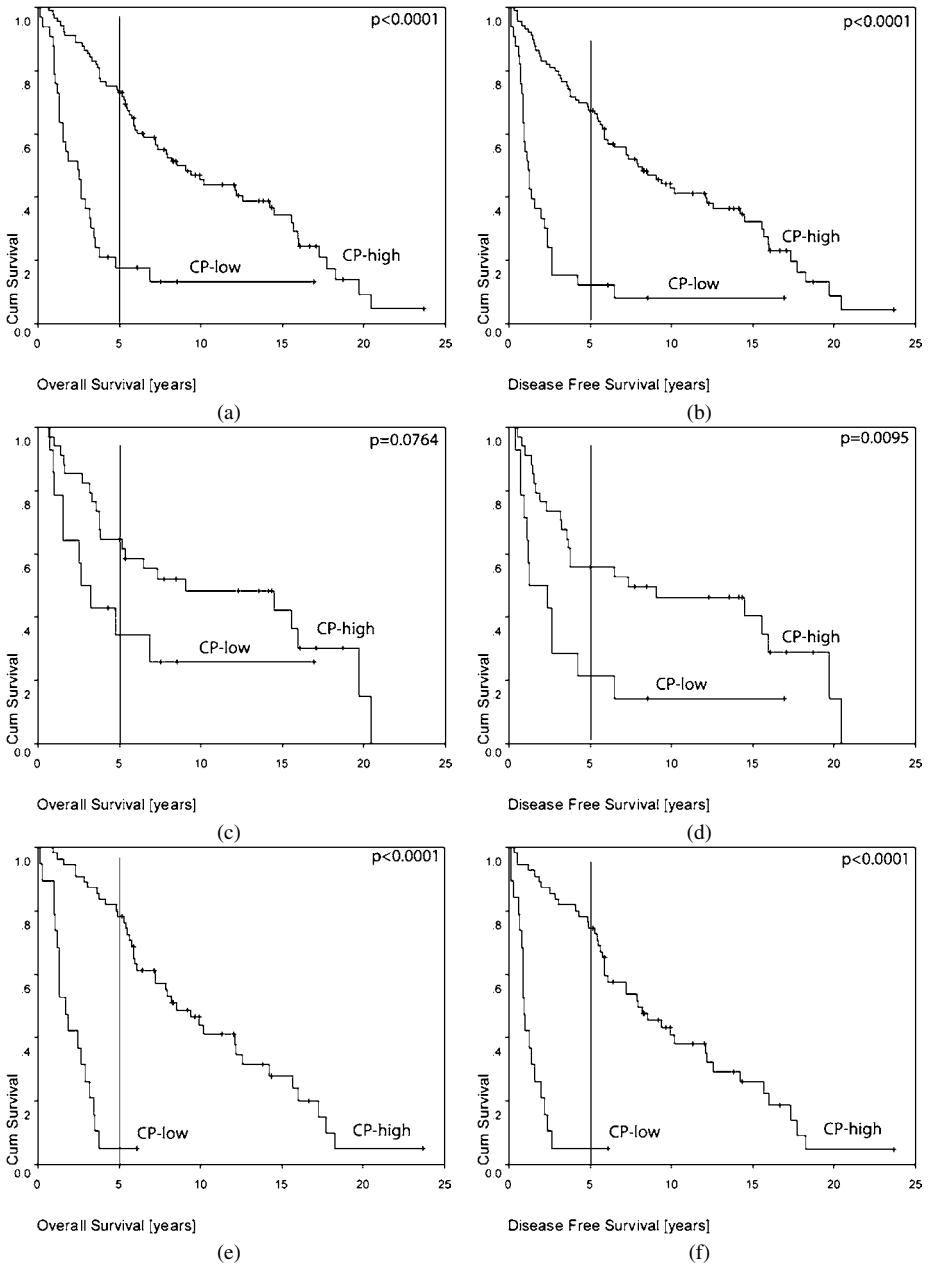



Fig. 4. Kaplan–Meier curves of the *combined series* for CP-low and CP-high patients: (a) OS and (b) DFS of the complete set of patients, (c, d) represent the OS and DFS of the left sided tumors and (e, f) of the right sided tumor. The dashed line indicates the 5-year survival time.

1.98; DFS $p = 0.0095$, HZ 2.51). OS and DFS were not significantly different for LN status but for DFS tumor status and stage differed significantly. However disease specific survival (DSS) did show significant values for all parameters (Table 3).

(b) Eighteen (24%) of the right sided patients had a low CP and 56 (76%) a high CP. Survival analysis using Kaplan–Meyer showed highly significant values for OS and DFS (OS $p < 0.0001$, HZ 9.56; DFS $p < 0.0001$, HZ 9.14). Five year survival rates (OS/DFS) for CP-low compared to CP-high patients were respectively 5.3%/5.3% and 78.2%/74.5%. Significant differences for the TNM parameters were found, respectively for LN status and for stage, but not for DFS for tumor status. See Tables 3, 4 and Fig. 4c–f. We conclude that CP is of prognostic value for patients with either a left or right sided tumor, although for patients with a right sided tumor this is more evident.

*3.5.2. MSI status*

Twenty-three (18.9%) out of 122 patients showed abrogation of MLH1 and PMS2 and were MSI-H. One MSI-H patient had a colon carcinoma located left sided and 22 patients right sided.

Two patients with right sided tumors had a low CP. For MSI-H the five year survival rates for OS and DFS for CP-low patients compared to CP-high were respectively 0%/0% and 81.5%/76.6%.

Excluding the MSI-H tumors from analysis resulted in identical data for OS and DFS (OS $p < 0.0001$, DFS $p < 0.0001$ for both series). Since both series were not significantly different with respect to CP values ($p = 0.3$), OS ($p = 0.3$) and DFS ($p = 0.3$) we can conclude that these results indicate that the prognostic power of CP remained independent of MSI status.

*3.6. Relation with tumor stage*

Twenty-six patients were classified as stage I, 34 stage IIA, 6 stage IIB and respectively 8, 31 and 16 stage IIIA, B or C (Table 1).

The mean OS for CP-low versus CP-high for stage I and II patients was 3.96 years (range 1.30–6.62) and 10.33 years (range 8.80–11.86) ($p = 0.026$). For DFS this was 3.74 years (range 1.93–5.56) and 9.93 years (range 9.64–12.92) ($p = 0.0007$).

The mean OS for CP-low versus CP-high for stage IIIA–C patients was 3.85 years (range 1.12–6.58) and 9.61 years (range 8.04–11.19) ($p = 0.076$). For DFS this was 2.13 years (range 0.88–3.38) and 9.73 years (range 6.67–12.79) ($p < 0.0001$).

These results indicate that CP can be a discriminative parameter for as well low as high staged patients.

## 4. Discussion

The carcinoma-stromal composition is an important prognostic parameter as is proven in the presented studies in patients with stage I–III colon cancer. The determined carcinoma percentage (CP) classification can easily be applied in routine pathology in addition to the TNM classification to select patients with increased risk for recurrence of disease. Although statistical analysis of two independent series proved that CP is an independent parameter, we realize that the series that were analyzed are relatively small.

The use of adjuvant therapy for stage II patients remains controversial, and the identification of reliable prognostic factors may aid therapeutic decision-making. In our study we noticed a high number of patients with a low carcinoma percentage (CP-low) depending on stage, from 7.7% in stage I to 68.7% in stage IIIC patients. For stage I, II patients OS and DFS was significantly lower for patients with CP-low compared to patients with CP-high; 3.96/3.74 years versus 10.33/9.93 years (OS $p = 0.0255$, DFS $p = 0.0007$).

Three out of 4 (75%) stage IIa patients with CP-low died within 5 years due to their disease and 5 out of 30 (17%) patients with CP-high died within 5 years (sensitivity 37.5%, specificity 96.2%). For stage III patients, 22 out of 26 (96%) with CP-low died within 5 years due to their disease and 14 out of 29 (64%) patients with CP-high died within 5 years (sensitivity 61%, specificity 70%). Although the sensitivity is quite low, the specificity is very high and therefore CP-low in stage II patients could be indicative for adjuvant therapy or better-individualized treatment for an additional group of patient. In contrast, for stage III patients the sensitivity is too low and would result in undertreatment of patients.

Notably, in Northern European countries for stage II patients standard treatment does not include adjuvant treatment with chemotherapy, although for high risk patients the ESMO (European Society for Medical Oncology) recommends adjuvant treatment. In a recent study treatment with FOLFOX resulted in a relative reduction on risk of recurrence of 28% for high risk patients [16,17].

Our results for stage II patients are encouraging, nevertheless we should confirm our results in a much larger patient set. Our future research is directed to this goal.

Furthermore we observed that for the patients with a low CP the T stage is of less importance and that there-

fore these tumors might have a different mechanism for metastasizing.

Invasion and metastasis of colorectal cancers include various steps, such as proteolysis, adhesion, angiogenesis and cell growth, for which many genes have been identified [18]. In the proteolysis step, proteinases, which are produced by cancer cells but also by fibroblasts, degrade extracellular matrix (ECM) components and enable cancer cells to detach from the primary site [19]. In our study an increase of stromal cells in the primary tumor correlated significantly with poor prognosis. Malignancy emerges from a tumor-host microenvironment in which the host participates in the induction, selection and expansion of the neoplastic cells [20]. The stromal matrix has been shown to influence epithelial cell function in both malignant behavior and nonmalignant differentiation [21]. Stromal cell activation may be reflected in modifications of the adjacent ECM that are favorable to the microinvasion of cancer cells. This phenomenon could explain our findings.

A variety of cell types populate the stromal compartment, such as lymphocytes, granulocytes, fibroblasts and endothelial cells. The relative abundance of each cell type may change at the local site of tumor cell invasion [22,23].

Cancer cells expressing adhesion molecules are more likely to adhere to the ECM, leading to subsequent invasion and metastases. A prominent example is the epithelial-to-mesenchymal transition (EMT) during the process of wound healing in which cells loosen their intimate cell–cell contacts and acquire mesenchymal properties which means that epithelial cells can be converted into fibroblast-like cells. Cancer cells undergoing EMT develop invasive and migratory abilities. EMT of cancer cells is increasingly being recognized as an important determinant of tumor progression but also fibroblasts are implicated to play a role in metastasis [12,24]. A prominent factor to induce EMT is the transforming growth factor-$\beta$ (TGF-$\beta$), which mediates fibroblast activation during wound healing [25]. For microarray analysis of gene expression patterns a wound-response signature is already known for breast cancer patients showing improved risk stratification for a poor prognosis independently of known clinicopathologic features [13].

Data for microsatellite instability (MSI) and chromosomal instability (CIN) have demonstrated that these groups are characterized by a different clinical outcome; tumors originating from the right colon have a better prognosis than tumors from the left part due to a high percentage of MSI-H lesions. In a publication by Gervaz et al. it was even stated that clinical decision making regarding adjuvant therapy might be stratified in the future according to MSI status of cancer [26]. Tumors with MSI-H rarely metastasize, neither locally, nor distant, have a more favorable stage and have been repeatedly reported as a favorable prognostic marker [27,28]. In our study we have excluded HNPCC patients, therefore patients were only tested for sporadic MSI-H using immunohistochemical staining for MLH1 and PMS2, this combination confirms the abrogation of the MLH1 protein for all MSI-H sporadic tumors. For MSI-H patients we found significant differences in OS and DFS when CP was added as additional parameter: 0%/0% versus 81.5%/76.6%.

We observed a difference between left and right sided tumors. For tumors located right sided in the colon, significant differences were found for CP, but also for LN status and stage but less for tumor status. For the left sided tumors, CP was a significant prognostic factor. All other TNM parameters did not reach significance for OS, only DFS for tumor status and stage were significantly different. However, disease specific survival (DSS) did show significance for all parameters.

As far as we know, no data are published about the influence of the carcinoma-stromal proportion on outcome in primary colon tumors. Although many pathologists will recognize the feature, the impact on prognosis was not known by now. Our study describes a candidate parameter that after proper training could be used in routine diagnosis, in addition to the TNM classification, to further stratify in more individual risk.

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# Chapter 6

## Presence of a high amount of stroma and downregulation of SMAD4 predict for worse survival for stage I-II colon cancer patients

*Submitted*

# Presence of a high amount of stroma and downregulation of SMAD4 predict for worse survival for stage I-II colon cancer patients

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**Abstract.** *Purpose:* For stage I-II colon cancer a significant number (5%-25%) of patients has recurrent disease within 5 years. There is need to identify these high-risk patients as they might benefit from additional treatment. Stroma-tissue surrounding the cancer cells plays an important role in the tumor behavior. The proportion of intra-tumor stroma was evaluated for the identification of high-risk patients. In addition, protein expression of markers involved in pathways related to stroma production and epithelial-to-mesenchymal transition (EMT) was analyzed: β-catenin, TGF-β-R2 and SMAD4. *Methods:* In a retrospective study of 135 patients with stage I-II colon cancer, the amount of stroma was estimated on routine haematoxylin-eosin stained histological sections. Sections were also immunohistochemically stained for β-catenin, TGF-β-R2 and SMAD4. *Results:* Of 135 analyzed patients 34 (25.2%) showed a high proportion of stroma (stroma-high) and 101 (74.8%) a low proportion (stroma-low). Significant differences in overall-survival and disease-free-survival were observed between the two groups, with stroma-high patients showing poor survival (OS $p<0.001$, HZ 2.73; DFS $p<0.001$, HZ 2.43). A high-risk group was identified with stroma-high and SMAD4 loss (OS $p=0.008$, DFS $p=0.005$); 12 of 14 (85.7%) patients died within 3 years. In a logistic-regression analysis a high proportion of stroma and SMAD4 loss were strongly related (HZ 5.42, CI 2.13-13.82, $p<0.001$). *Conclusion:* Conventional haematoxylin-eosin stained tumor slides contain more prognostic information than previously fathomed. This can be unleashed by assessing the tumor-stroma ratio. The combination of analyzing the tumor-stroma ratio and staining for SMAD4 results in an independent parameter for confident prediction of clinical outcome.

## 1. Introduction

The five year survival rate for coloncancer stage I-II patients (AJCC staging) is 93% for stage I, 85% for stage IIa and 72% for stage IIb.[1] The high surgical cure rate for patients with 'low-risk" stage II and the outcome of clinical trials and meta-analysis give debatable recommendations for or against adjuvant chemotherapy.[2-6] For Northern European countries the current advice by the ESMO (European Society for Medical Oncology) is "no adjuvant treatment". Nevertheless 5%-25% of stage I-II patients will have recurrence of disease within 5 years.[1] Therefore there is

a strong need for additional parameters to select patients for additional therapy.
Pathological characterization as recommended by the ASCO (American Society of Clinical Oncology) serves as an indication for chemotherapy for "high risk" stage II patients, identified on the basis of clinical features as T4, obstruction or perforation and low number of removed lymph nodes (n<12).[7]

Recent models on metastatic pathways which focus on invasion include the "tumor-host" interface and in particular focus on the role of the stroma tissue. The proportion and the composition of tumor stroma differ between tumors, and are distinct from normal tissue stroma. [8]
A number of key parameters involved in intra-tumor stroma production that may support our finding can be found in the transforming growth factor–β (TGF-β) and Wnt-signaling pathway.
For the Wnt-signaling pathway, the main oncoprotein in colorectal cancer is the Wnt pathway effector β-catenin. Accumulation in the nucleus of β-catenin is indicative of activation of the *wnt*-signaling pathway through mutation of the APC-gene, which occurs at an early step of colorectal carcinogenesis.[9,10] Loss of membraneous E-cadherin in adherens junctions results in translocation of β-catenin from adherens junctions to the nucleus which in turn triggers the loss of E-cadherin and subsequently the EMT (epithelial-mesenchymal transition). β-catenin nuclear staining was found upregulated in the invading area of colorectal cancer and seems to correlate with metastasis and poor survival. [11]
For the TGF-β pathway, growth factors produced by tumor cells, cause the tumor surrounding stroma to become "reactive" upon which tumor cell proliferation, migration and angiogenesis is promoted. A molecular mimicry in tumor resembles stroma injury, which occurs in wound healing. Amongst others the TGF-ß signaling is a key regulator of this process.[12] Fibroblasts –the main cell type in stroma- may differentiate into so-called cancer-associated fibroblasts (CAFs) during the progression to invasive carcinoma.[13,14] EMT is engaged by several cytokines associated with proteolytic digestion of the basal membrane (by metalloproteinases) upon which the epithelium resides. The role of the TGF-β signaling pathway relates to both the primary tumor and the stroma. In addition, its role is dual: in early stages it blocks tumor growth, whereas in progressed stages it stimulates invasion and metastasis.[15] TGF-ß exerts its function by binding to specific transmembrane receptors, for which receptor II is found mutated in colorectal cancer.[16]
Smad proteins are key signal-transducers of the TGF-β pathway and are essential for the growth suppression function of TGF-β.[17] Smad proteins are regulators of transcription and act as tumor suppressor molecules whose inactivation by mutation or silencing is associated with pancreatic and colon cancer. For colon cancer, SMAD4 coded at 18q21.1, plays a key role; 18q deletion is observed in 30% of invasive colorectal carcinoma.[18-20]
In a former study we have investigated the proportion of intra-tumor stroma, on haematoxylin-eosin (H&E) stained histological sections, as prognostic parameter for colon carcinoma. In a set of 122 patients (stage I-III) a significant difference in survival time was observed between patients with a high amount of intra-tumor stroma (stroma-high) and patients with less

Table 1
Patient characteristics

| | Total | Stroma-high | Stroma-low |
|---|---|---|---|
| **Gender** | N (%) | N (%) | N (%) |
| **Male** | 74 (54.4) | 20 (57.1) | 54 (53.5) |
| **Female** | 61 (45.2) | 14 (41.2) | 47 (46.5) |
| **Mean age (yrs)** | 68.2 | 68.5 | 68.0 |
| **Location tumor** | | | |
| **Left** | 63 (46.7) | 22 (61.1) | 43 (42.6) |
| **Right** | 72 (53.3) | 14 (38.9) | 58 (57.4) |
| **T status** | | | |
| **T1** | 4 (3.0) | 1 (3.0) | 3 (3.0) |
| **T2** | 84 (62.2) | 23 (67.6) | 61 (60.4) |
| **T3** | 41 (30.4) | 10 (29.4) | 31 (30.7) |
| **T4** | 6 (4.4) | 0 | 6 (5.9) |
| **Stage** | | | |
| **I** | 24 (17.8) | 2 (5.9) | 22 (21.8) |
| **IIA** | 105 (77.8) | 32 (94.1) | 73 (72.3) |
| **IIB** | 6 (4.4) | 0 | 6 (5.9) |
| **Grading (differentiation)** | | | |
| **Well** | 23 (17.0) | 6 (17.6) | 17 (16.8) |
| **Moderate** | 77 (57.0) | 22 (64.7) | 55 (54.5) |
| **Poor** | 31 (23.1) | 6 (17.7) | 25 (24.8) |
| **Unknown** | 4 (2.9) | 0 (0) | 4 (3.9) |
| **MSI** | | | |
| **MSS** | 108 (80.0) | 32 (94.1) | 76 (75.2) |
| **MSI-H left sided** | 2 (1.5) | 0 | 2 (2.0) |
| **MSI-H right sided** | 23 (17.0) | 2 (5.9) | 21 (20.8) |
| **Unknown** | 2 (1.5) | 0 | 2 (2.0) |

stroma (stroma-low). Stroma-high patients showed a significantly worse survival.[21]
The current study is based on stage I-II patients only, to identify a subgroup of patients with bad prognosis who might benefit from adjuvant therapy. We have analyzed 135 stage I-II colon cancer patients with at least 11 years of follow-up for the proportion of tumor related stroma and for TGF-β-R2, SMAD4 and β-catenin. It was found that in particular SMAD4 allows for further prognostic stratification of stage I-II colon cancer patients.

## 2. Methods

### Patient recruitment

We included 139 colon cancer patients with stage I-II tumors (clinically staged accord-

ing to the classification of the AJCC)[22], who underwent curative surgery at the Leiden University Medical Center between 1980 and 2001. Fifty-eight patients were part of two consecutive series formerly published for H&E analysis only.[21] For this study, additionally 77 patients were obtained from a case-control series.

Case control series: Cases (n=27) considered with regional or distant recurrent disease between three months and five years after the date of diagnosis of primary colon carcinoma. Regional metastases were considered intra-abdominal or intrapelvic metastases in lymph nodes or in connective tissue. Fifty controls were selected with no locoregional or distant disease within five years after diagnosis of primary colon cancer. For each case two controls were matched for TNM stage, date of incidence and date of birth. None of the patients had pre- or postoperative chemo- or radiation therapy. Patients with synchronous second tumors, other malignancies in the past and death or recurrence (distant or loco-regional) within 1 month, were excluded.

All samples were handled in a coded fashion, according to National Ethical Guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies).

**Histopathological protocol**

Pathological examination entailed routine microscopic analysis of 5 µm H&E stained sections of the primary tumor. The amount of intra-tumor stroma, visually scored by three investigators (VS, KvL, WM), was estimated on the most invasive part of the tumor. For a detailed protocol see Mesker et al.[21] For the identification of microsatellite instability-high (MSI-H) patients, 5 µm slides were immunohistochemically stained for MLH1 and PMS2 markers.[23]

**Immunohistochemical staining for TGF-β-R2, SMAD4 and β-catenin**

Four-micron-thick sections from paraffin embedded tissue were positioned onto silane-treated Starfrost slides (Klinipath, Duiven, Netherlands) and left to dry overnight. Antigen retrieval was performed at low pH 6.0 citrate buffer (0.01 M) for TGF-β-R2 and β-catenin and at high pH 9.0 Tris 0.01M/EDTA buffer (0.001M) for SMAD4 for 10 min. Subsequently, slides were incubated at room temperature for 15 min (TGF-β-R2) or overnight (SMAD4, β-catenin) using antibodies to TGF-β-R2 (rabbit polyclonal antibody, ab28383, prediluted; Abcam, Cambridge, United Kingdom), SMAD4 (mouse monoclonal antibody, sc-7966, dilution 1:400 in 5% non-fat milk in PBS; Santa Cruz Biotechnology, Santa Cruz, CA) or β-catenin (mouse monoclonal antibody, clone 14, dilution 1:1600 in 1% PBS/BSA BD Biosciences Transduction Laboratories, Lexington). After the primary antibody step, slides were incubated for 30 min with EnVision-horseradish peroxidase anti-mouse or anti-rabbit (Dako-Cytomation, Heverlee, Belgium) followed by incubation with diaminobenzidine (liquid DAB+ Substrate Chromogen System, K3468, DakoCytomation, Heverlee, Belgium) for 5 min.

Control specimens were processed without primary antibodies. Internal positive control for SMAD4 consisted of normal epithelium and stroma for TGF-β-R2.

The intensity and pattern of the immunohistochemical staining was visually evaluated. In case of TGF-β-R2 membranous staining, four categories were applied;

from negative (0) to positive (3). For nuclear SMAD4 staining we used three categories (0=negative, 1=positive and 2=mixed (neg/pos) and for β-catenin four categories from membranous (0) to all nuclear expression (3)

**Statistics**

Statistical analysis was performed using SPSS software version 14.0. Overall-Survival (OS) was defined as the time period between the date of primary surgery and the date of death from any cause or the date of last follow-up.

Metastases-Free-Survival (MFS) was defined as the time period between the date of primary surgery and the date of first loco-regional or distant metastases or the date of last follow-up. Disease-Free-Survival (DFS) was defined, according to proposed guidelines, as the time from the date of primary surgery until the date of death or to the date of first loco-regional or distant recurrence or the date of a second primary tumor.[24]

Stroma-high was defined as: < 50% tumor cells including the values 10, 20, 30 and 40% tumor and stroma-low: ≥ 50% tumor cells including the values 50, 60, 70, 80 and 90%.

Analysis of the survival curves was performed using Kaplan-Meier Survival Analysis and differences in survival distributions were tested using Log Rank Statistics. The Cox proportional hazards model was used to determine the Hazard Ratio (HZ) of explanatory variables on OS and DFS. The logistic regression analysis was used to determine the interaction between the variables intra-tumor stroma and SMAD4.

Of the various staining patterns the following categories were statistically evaluated: TGF-β-R2 0 versus 1,2,3; SMAD4 0 versus 1,2; β-catenin 0,1 versus 2,3.

## 3. Results

**Patient demographics**

Four of the 139 selected patients were rejected on the basis of poor quality of the histological material, leaving 135 patients for analysis.

The study consisted of 74 men (54.8%) and 61 women (45.2%), with a mean age of 68.2 years (SD 11.5; range 30.1-85.0 years).

From 135 primary tumors 63 (46.7%) were located left sided and 72 (53.3%) right sided. Left sided tumors were defined as: flexura lienalis n=3), colon descendens (n=5), colon sigmoideum (n=43) and rectosigmoideum (n=12), and right sided as: coecum (n=34), colon ascendens (n=15), flexura heptica (n=10) and colon transversum (n=13) (Table 1).

**Histopathology**

Routine H&E stained sections from the most invasive part of the tumor were microscopically analyzed for the presence of stroma involvement using 5x and 10x microscope objectives. The variation in scoring for the individual pathologists for stroma-high versus stroma-low was 6.9% (range 4.4–8.8%) with low inter-observer variation between the three pathologists ($p< 0.0001$, Kappa <0.0001).

**Correlation with prognosis**

Of 135 analyzed patients 34 (25.2%) were scored stroma-high and 101 (74.8%) stroma-low. Significant differrences were found for overall (OS), disease free (DFS) and metastasis free (MFS) sur-

Table 2
P values (univariate) for stroma-high versus stroma-low patients and TNM parameters defined per site.

| **Univariate** | **Total** n=135 | **Left** n= 63 | **Right** n= 72 |
|---|---|---|---|
| **Stroma** | | | |
| OS | <0.001 | 0.001 | 0.001 |
| DFS | <0.002 | 0.002 | 0.007 |
| MFS | <0.001 | | |
| **HZ** | | | |
| OS | 2.73 | 2.85 | 2.99 |
| DFS | 2.43 | 2.63 | 2.50 |
| **95% Conf. Int.** | | | |
| OS | 1.73-4.30 | 1.52-5.33 | 1.49-6.00 |
| DFS | 1.55-3.82 | 1.42-4.90 | 1.26-4.97 |
| **T- status*** | | | |
| OS | 0.772 | 0.006 | 0.396 |
| DFS | 0.632 | <0.001 | 0.550 |
| **Stage**** | | | |
| OS | 0.752 | 0.685 | 0.387 |
| DFS | 0.895 | 0.693 | 0.502 |

* T2 versus T3: p= 0.47 ** Stage I versus IIa + IIb: p= 0.84, Stage I versus IIa: p= 0.79.

Table 3
Characteristics of immunostaining for TGF-β-R2, SMAD4 and β-catenin in relation to the amount of intra-tumor stroma of the primary tumor.

| | **TGF-β-R2 *** N= 117 | | **SMAD4*** N=118 | | **β-catenin*** N=117 | |
|---|---|---|---|---|---|---|
| | **negative** | **positive** | **negative** | **positive** | **nuclear** | **membrane** |
| **Stroma-H** | 2 (2%) | 26 (2%) | 14 (2%) | 14 (12%) | 18 (15%) | 11 (9%) |
| **Stroma-L** | 11 (9%) | 78 (67%) | 14 (12%) | 76 (64%) | 41 (35%) | 47 (40%) |
| Chi-square | p= 0.444 | | p< 0.001 | | p= 0.148 | |

* Percentage is based on the total number for markers analyzed patients.

Table 4
Results of SMAD4 staining relative to the amount of stroma in the primary tumor.

| | | Stroma high* | Stroma-low** |
|---|---|---|---|
| **SMAD4-negative** | Percentage of patients | 11.9% | 11.9% |
| | Percentage at 5-year | **7.1%** | 85.7% |
| **SMAD4-positive** | Percentage of patients | 11.9% | 64.4% |
| | Percentage at 5-year | 57.1% | 80.3% |

Percentage is based on the total number of patients that were analyzed for markers (n=118).
* Significance for Smad staining within the stroma-high group: OS p=0.008 DFS p=0.005.
** Significance for Smad staining within the stroma-low group: OS p=0.937 DFS p=0.685.
Note: The series that was analyzed consists partly of a consecutive and partly of a case-control set. Calculated hazard ratio's (HZ) are valid and meaningful but the 5-year survival time can not be used to generalize. In our data set 30% of the patients had a recurrence within 5 years; the actual rate for stage I-II patients is 25%.

vival between stroma-high and stroma-low patients (OS p<0.001; HZ 2.73, DFS p=0.001; HZ 2.43, MFS p<0.001) (Table 2, Supplementary Table 1 and Figure 1).

For stage IIa, 32 (30.5%) out of 105 patients were scored stroma-high. Of these, twenty-one (65.6%) patients died within 5 years and 11 (34.4%) were still alive after 5 years (OS p<0.001; HZ 2.7 (range 1.64-4.45), DFS p=0.001; HZ 2.30 (range 1.41-3.74)). Twenty of 21 patients died due to their disease, 15 developed metastases to the liver, 4 to the peritoneum and one to the lung.

Remarkably, none of the 21 "high risk" patients (defined as stroma-high, death ≤ 5 years) fulfilled the ASCO "high risk" criteria for T4, obstruction or perforation.

Six patients with stage IIb were included in this series. All patients were scored as stroma-low. The mean survival for these patients was OS: 6.39 years, DFS 6.08 years.

For stage I, 8.3% (n=2) of the patients were scored as stroma-high. The survival for these two patients was respectively: OS/DFS both 2.67 years, 2.08/0.89 years. The mean overall survival time for the stroma-low group was 10.8 years.

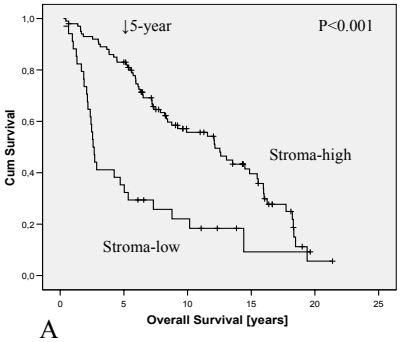
Within all stages no correlation was observed between the proportion of stroma and the tumor differentiation grade (ASCO recommendations).

**Topography**

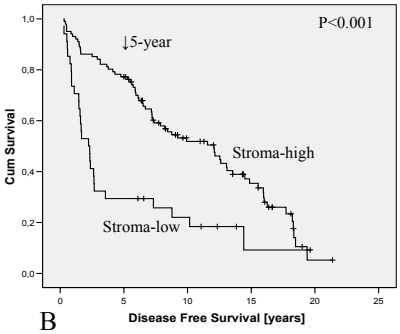
We investigated the topography separately, known that this parameter effects prognosis. From a total of 135 patients 46.7% (n=63) had a tumor located left sided in the colon and 53.3% (n=72) right sided.

Twenty (31.7%) of the left sided tumors were stroma-high and 43 (68.3%) stroma-low. Survival analysis showed significant differences between both groups (OS p<0.001; HZ 2.85, DFS p=0.002; HZ 2.63) (Table 2 and Supplementary Table 1). Fourteen (19.4%) of the patients with a right sided tumor were stroma-high and 58 (80.6%) stroma-low. Significant differences between both groups were observed (OS p=0.001; HZ 2.99, DFS p=0.007; HZ 2.50).

Figure 1




A

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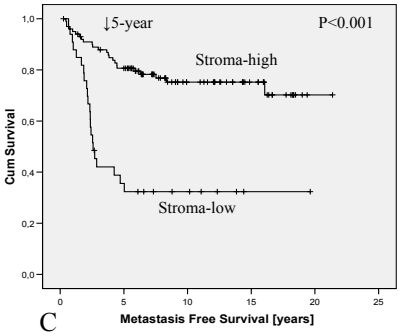
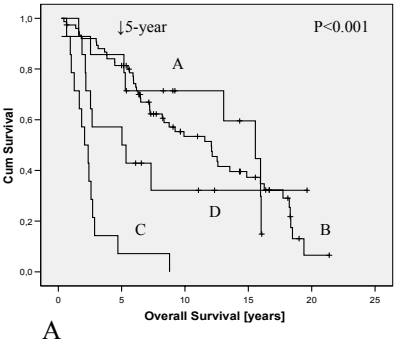


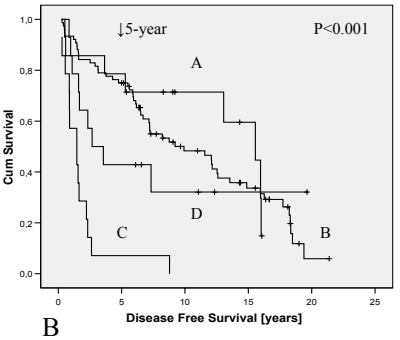

C

Figure 1.
Kaplan-Meier survival curves for stroma-high and stroma-low patients: (a) OS, (b) DFS, (c) MFS.
Notably, the mean age of the analyzed patient group was 68.2 years with a mean follow-up time of 10.9 years. As some patients have a follow-up period of 20 years the full survival time was displayed.

Figure 2




A




B

Figure 2.
Kaplan-Meier survival curves for stroma-high patients and stroma-low patients with positive and negative SMAD4 staining: (a) OS, (b) DFS.
Notably, the mean age of the analyzed patient group was 68.2 years with a mean follow-up time of 10.9 years. As some patients have a follow-up period of 20 years the full survival time was displayed.
A. Stroma-low / SMAD4-negative.
B. Stroma-low / SMAD4-positive.
C. Stroma-high / SMAD4-negative.
D. Stroma-high / SMAD4-positive.

Although the number of patients with stroma-high differed per location (left or right), the prognosis for stroma-high patients was similar: HZ 2.85 versus 2.99 and HZ 2.63 versus 2.50.
Twenty-five patients were MSI-H of which 23 (92%) were located right sided and 2 (8%) left sided. Five-year survival for the total MSI-H group was 90% compared to the microsatellite stable (MSS) group with 70%.

**Immunostaining for TGF-β receptor**
Staining for TGF-β-R2 resulted in a positive membranous staining of the tumor cells. When no membranous staining was observed it was concluded that TGF-β-R2 was abrogated. From 117 patients stained for TGF-β-R2, 104 (88.9%) showed positive membranous expression and 13 (11.1%) were negative. No significant difference in survival time was observed between both groups (OS p=0.079, DFS p=0.106).
Between the stroma-high and stroma-low group no significant difference in survival times were observed for patients with and without abrogation of TGF-β-R2 (Table 3).

**Immunostaining for β-catenin**
Staining for β-catenin resulted in membranous staining, nuclear staining, or showing both; these patients were counted as nuclear staining. The number of patients with nuclear staining was 59. There was no significant difference in survival time for patients with and without nuclear expression (OS p=0.227, DFS p=0.116).
From 117 patients stained for β-catenin 29 were stroma-high of which 18 (62.1%) had expression of the protein in the nucleus and 11 (36.7%) showed expression in the cytoplasmic membrane.
No significant correlation was observed between the stroma-high and stroma-low group and either nuclear or membraneous β-catenin expression (Table 3).

**Immunostaining for SMAD4**
In case of active TGF-β signaling, SMAD4 positive nuclear staining is expected. Nuclear and cytoplasm negative staining indicates abrogation of the SMAD4 gene expression leading to changes in the TGF-β pathway. From 118 patients stained for SMAD4, positive nuclear staining was seen in 90 cases, 17 were negative and 11 patients showed both positive and negative areas within the tumor; these latter patients were counted as negative.[25]
The total number of patients with negative staining for SMAD4 was 28 (23.5%). There was a significant difference in survival time between the SMAD4 positive and the SMAD4 negative patients (OS p=0.006, DFS p=0.022). The proportion of SMAD4 positive and SMAD4 negative patients within the stroma-high group was about equal but a distinct difference in survival time between both groups was observed, with stroma-high/SMAD4-negative patients showing a worse prognosis (OS p=0.008, DFS p=0.005).
Twelve of the 14 (85.7%) stroma-high /SMAD4-negative patients died within 3 years. For the stroma-low group this difference was not significant (OS p=0.937, DFS p=0.685). Percentages of 5 year follow up were 7.1% for stroma-high/SMAD4-negative patients and 80.3% for stroma-high/SMAD4-positive patients (Tables 3 and 4).
Combined use of H&E staining and SMAD4 immunohistochemistry as prognostic marker (stroma-high/stroma-low with positive or negative staining of

SMAD4) showed significantly different Kaplan-Meier curves (OS $p<0.001$, DFS $p<0.001$) (Figure 2).
A group of "high risk" patients with low survival time showing a high amount of intra-tumor stroma and negative SMAD4 staining could be distinguished with additional independent prognostic value.
In a multivariate cox-regression analysis, the amount of stroma appeared to be an independent factor for survival ($p<0.001$, HZ 2.73).
In a logistic regression analysis the interaction between the variables high intra-tumor stroma and loss of SMAD4 were found to be strongly related (HZ 5.42, CI 2.13-13.82, $p<0.001$) indicating that SMAD4 staining can be a specific marker to select "high risk" patients.
No significant relationship between the amount of stroma and β-catenin staining or the amount of stroma and TGF-β-R2 was found.

## 4. Conclusions

In a former study we investigated the tumor-stroma ratio as prognostic parameter for stage I-III colon cancer patients. Significant differences in survival time were found for patients showing different amounts of intra-tumor stroma within the primary tumor.[21] Patients with a high percentage of stroma were found to have a worse prognosis.
In the current study we focus on stage I-II patients aiming at the identification of a subgroup who might benefit from additional therapy. Additionally, we investigated three elements involved in the signaling pathways related to tumor stroma interactions: TGF-β-R2, SMAD4 and β-catenin. The already strong prognostic information provided by the tumor-stroma ratio was further refined by adding information regarding the SMAD4 status, which loss selects for a specific group of patients with more aggressive tumors. This specific group of patients with stroma-high/loss of SMAD4 showed a low 5-year survival of 7.1% compared to 80.3% for patients with stroma-low/SMAD4 positive staining.
Several studies report a higher frequency of SMAD4 inactivation in patients presenting unfavorable survival, which is in agreement with our observations.[19,26,27]
Although other groups give evidence that increased nuclear β–catenin expression is independently associated with higher N stage and worse survival,[28,29] we did not find β-catenin to correlate with either overall survival or associated with stroma involvement. This finding is in line with a multivariate analysis of adhesion molecules for stage II colorectal tumors performed by Ngan et al where E-cadherin and CD44 were found more informative than β–catenin.[30]

Currently there is no univocal policy for standard treatment of stage II patients. Treatment of the complete group is not meaningful, although for high-risk patients, the ASCO recommends adjuvant treatment.[31] A recently published paper by the QUASAR Collaborative Group reports that treatment of this group would result in an absolute benefit from an 18% reduction in mortality of 5.4% for high-risk patients compared to 3.6% in low risk patients.[32]
According to literature 25% of colon cancer stage II patients have recurrence within 5 years.[1] Within our analyzed group this percentage was 30%. Of the patients with a high amount of stroma, 62% had

recurrence of disease, whereas for patients with stroma-high in combination with SMAD4 abrogation this was 86% within 5 years.

These results show that tumor-stroma ratio as single parameter or in combination with SMAD4 immunohistochemistry can further select for a patient population with specific bad prognosis. When confirmed in series from other institutions our approach might contribute to a better selection of high risk stage I and II patients that might benefit from adjuvant treatment. Consequently, prospective studies to select patients for a randomized clinical study in which adjuvant therapy is selectively applied in stage I and II colorectal cancer should follow.

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Supplementary Table 1.
Mean and median survival data.

| | | N | OS / DFS (yrs) | 95% confidence interval (CI) |
|---|---|---|---|---|
| **Total series** | | | | |
| Stroma-low | Mean | 101 | 11.7 / 10.9 | 10.3-13.0 / 9.5-12.2 |
| | Median | | 12.2 / 12.1 | |
| Stroma-high | Mean | 35 | 5.8 / 5.2 | 3.7-7.9 / 3.0-7.4 |
| | Median | | 2.6 / 2.2 | |
| **Left sided** | | | | |
| Stroma-low | Mean | 43 | 12.5 / 11.7 | 10.4-14.7 / 9.4-13.9 |
| | Median | | 15.6 / 14.5 | |
| Stroma-high | Mean | 20 | 5.7 / 5.1 | 3.0-8.4 / 2.3-7.9 |
| | Median | | 2.7 / 2.2 | |
| **Right sided** | | | | |
| Stroma-low | Mean | 58 | 10.9 / 10.2 | 9.3-12.5 / 9.5-11.8 |
| | Median | | 11.6 / 9.9 | |
| Stroma-high | Mean | 14 | 5.1 / 4.6 | 2.7-7.5 / 2.0-7.2 |
| | Median | | 2.5 / 1.6 | |

# Chapter 7

Summary and General Discussion

Colorectal cancer (CRC) is one of the most frequently occurring cancers worldwide next to breast, prostate and lung cancer. In 2004, 2,260 men and 2,175 women died because of CRC. The incidence of CRC in The Netherlands is 9,898 cases per year and high compared to other countries. Due to an increasing life expectation of the population, the CRC incidence is expected to increase with 41.7% between the period of 2005–2025 (Dutch cancer registration databank). Therefore population screening for early detection has been started (see also introduction). Early detection using the FOBT (fecal occult blood testing) screening test reduces the risk of death due to colon cancer with 15-20% when the test is performed every 2 years, as was the result of large screening programs in the US and Europe.[1-4]

In addition to early detection, factors that influence the outcome of disease are of importance. Spread of disease is classified according to the Tumor-Node-Metastasis (TNM) classification and lymph node metastasis is the strongest prognostic parameter to select patients for adjuvant treatment schedules as radiotherapy and chemotherapy.

Stage I and II patients have negative lymph nodes ($T_x$,$N_0$,$M_0$) and the five year survival rate according to the AJCC (American Joint Committee on Cancer) for these patients is 93% for stage I, 85% for stage IIa and 72% for stage IIb. The current advice for therapy for Northern European countries by the ESMO (European Society for Medical Oncology) for these low stages is "no adjuvant treatment", thus surgery only.

Nevertheless 5%-25% of stage I-II patients, will have recurrence of disease within 5 years.[5] Therefore there is a strong need for additional parameters to select for patients in who might benefit adjuvant therapy.

For patients with advanced stages, the success of curation and expected prognosis of the patient after surgical treatment are dependent on the type of adjuvant treatment. Standard treatment for stage III patients (with positive lymph nodes) consists already for 15 years of 5FU+LV (5-fluorouracil + leucovourin), to which since 5 years Irinotecan and Oxaliplatin are added. These combinations of chemotherapeutics are currently being tested in clinical trials to which most recently also bevacizumab and cetuximab are added. A recently published paper by the QUASAR Collaborative Group describes the use of adjuvant treatment with fluorouracil and folinic acid, which reduces the 5-year mortality with 18% for stage III patients.[6]

For stage II patients, in common not adjuvantly treated, similar proportional reductions were observed in this study, but toxicity, costs and inconvenience of treatment hamper the treatment of all patients. Current recommendations are that patients with stage II disease who have a relatively higher average risk of recurrence (so called high-risk patients) –tumor stage T4, vascular invasion, low number of resected LN's (<12) or perforation or obstruction-chemotherapy should be offered.[6,7]

For the QUASAR study the high-risk group was defined as - tumor stage T4 or vascular invasion -. Assuming that the 5-year mortality without chemotherapy is 20%, treatment would result in an absolute benefit in survival of 5.4% for high-risk patients compared to 3.6% in low risk patients.[6]

According to the results of the QUASAR

study high-risk patients might benefit from additional treatment, but how can this group be identified?

In the introduction of this thesis many parameters were described to select patients at risk of disease recurrence for adjuvant therapy. Also the applicability of biomarkers that could predict the outcome of treatment with chemotherapy may aid to tailor-made individualized therapy. Therapy has impact on prognosis but without therapeutical consequence prognostic markers have less clinical value. For stage II colorectal cancer patients effectiveness of treatment with fluorouracil and folinic acid is reported below the 5% level and therefore not routinely applied.[6] Although of major importance, this thesis did not focus on therapy but on the evaluation of new parameters to select patients for additional therapy.

To identify novel markers in patients for additional treatment several methodologies are available such as expression- and genomic profiling, proteomics and others. However, in this thesis cell based microscopical parameters have been optimized and evaluated as prognostic markers to select patients for adjuvant treatment schedules and to predict clinical outcome. Automated microscopy has been applied to optimize the analysis of the TNM parameter lymph node involvement (N). A novel parameter was identified based on the proportion of stroma within the primary tumor stimulated by mediators within the TGF-β/SMAD4 pathway. Finally, genome profiling results are presented on differential genomic alterations of primary tumor tissue samples obtained from patients with and without disseminated tumor cells in their bone marrow.

A general introduction on colorectal cancer is presented in **chapter 1**.
Current routine pathology parameters are reviewed in the scope of staging and selecting patients at risk for developing recurrence of disease for additional treatment. Furthermore new biological and molecular markers and their role in relation to the current TNM classification are discussed.

Biological parameters such as the presence of micrometastases in lymph nodes, disseminated cells in bone marrow and circulating tumor cells in peripheral blood have shown to be of prognostic value and provide more insight in the process of dissemination and metastases as has been described in chapter 6 of this thesis.[8-13] However, the detection of these often rare occurring cells is being hampered by their low frequency. Visual evaluation of multiple lymph node sections or slides prepared from bone marrow or blood is tedious, time consuming and often irreproducible. Automated microscopy can fulfill this task with high accuracy. In **chapter 2** possibilities are discussed for the analysis of micrometastasis in lymph nodes, disseminated cells in bone marrow and circulating tumor cells in peripheral blood using automated imaging devices. These systems use software to identify cells based on their immunohistochemical or immunocytochemical staining pattern. Usually a pathologist performs visual verification of the identified cells and takes the final decision. In this chapter the performance is discussed of the current available equipment and the requirements for introducing this equipment in routine diagnostic practice.

In **chapter 3** automated microscopy as described in chapter 2 was applied to

improve detection of micrometastases in lymph nodes in stage II colorectal cancer patients and consequently to identify patients at high risk for recurrence. This study was performed using automated microscopy on paraffin embedded lymph node tissue of a patient group earlier studied by RT-PCR for CEA for the detection of tumor cells in histologically negative lymph nodes by Liefers et al.[14] They found a five-year survival rate of 50 percent for the micrometastasis-positive group and 91 percent for the negative group (P=0.02).
For automated microscopy on paraffin embedded lymph node tissue of the same patients, blocks were serially sectioned and immunohistochemically stained using antibodies against cytokeratin. For each lymph node 10 slides, cut at different intervals, with each containing five consecutive sections were analyzed using high throughput flatbed scanning. Comparable results were obtained as published by Liefers et al. which implies an upstaging for stage II patients to stage III. Based on these findings special software was developed for the ARIOL automated microscopy system (Applied Imaging, a Genetix company) and the same series was re-analyzed. This resulted in an similar sensitivity but in a more userfriendly method suited for routine applications. The advantage of automated microscopy over RT-PCR is that morphology remains intact for further evaluation or investigation of the cells.
The next part of the study addressed the question of how many slides should be investigated for an optimal diagnosis, taking theoretical as well as practical considerations into account. For each lymph node 10 sections with intervals of 200 µm were analyzed (same set of patients as in the former study). A number of 4 sections was found to be a good compromise between sensitivity and practical applicability. One could consider the implementation of the described techniques in routine pathology. However, since the number of surgically removed lymph nodes in colorectal cancer can be as high as 20 per patient (norm is >12 resected LN's) this approach could better be applied for detailed analysis of sentinel nodes only.

The presence of tumor cells in the bone marrow (BM) of cancer patients has shown to be associated with a worse prognosis, predominantly for patients with breast cancer.[8, 11, 12, 15-18]
To evaluate this parameter for colorectal cancer, collection of bone marrow aspirate was started from each new CRC patient at the LUMC in the year 2000. A standardized protocol was developed including aspiration of 5 ml of BM from both cristae, the preparation of slides containing 2 million cells per slide, monoclonal antibody staining for cytokeratin followed by automated microscopical analysis of the prepared slides. Till now 300 patients have been analyzed, with 15% of the patients showing tumor cells in the BM. **Chapter 4** describes the use of array-CGH to detect genomic alterations in primary tumor tissue from BM-positive patients compared to matched (regarding TNM stage and site) BM-negative patients. The goal of this study was to identify a specific genome profile that correlates with the occurrence of DTCs in the BM. A higher number of genomic alterations confirmed by interphase FISH was found in the BM-positive group as compared to the BM-negative group. Chromosomes 6 (p21.1), 9 (p11.2-p13.3), 12 (q13), and 16 and 19 (full chromosomes) were most frequently

gained for the BM-positive group, with losses for chromosome 11q (q22.3-q25) and 15q (q11.2-q12 and q14-q21). In this pilot study a distinct genome profile predominantly seen in BM-positive patients was identified; confirmation of this finding in a larger group of patients is however needed.

When applying array-CGH for tumor tissue analysis it is common practice to select those parts of the tissue in which tumor cells form the major component, as admixtures of abundant stroma and inflammatory cells will lead to masking of amplifications and deletions. Doing so, it was noticed that for the set of patients with bad prognosis the amount of tumor material was often less than 50% or even much lower. As these samples did not meet the criteria for a reliable array-CGH this set of patients was not eligible for analysis. The tumors of patients with a good prognosis showed the opposite; abundant tumor and less stroma. These observations led to the prognostic evaluation of the stromal proportion as an independent parameter in a larger patient series of which the results are presented in **chapter 5**. In a retrospective study of 122 patients (stage I-III) with at least 10 years follow up, 33 (27%) patients were stroma-high and 89 (73%) stroma-low. Five-year survival rates were respectively for OS: 15.2% and 73.0% and for DFS: 12.1% and 67.4 with stroma-high patients performing worse (OS $p<0.0001$, HZ 3.73; DFS $p<0.0001$, HZ 4.18). In a multivariate Cox regression analysis, the amount of stroma remained an independent variable when adjusted either for stage or for tumor status and lymph node status (OS $p<0.001$, DFS $p<0.001$).

It is known that the 18q chromosome is homozygously deleted in 75% of CRC. In this region the DCC gene is located at (q21.3)[19] The Transforming Growth Factor–β (TGF-β) pathway plays a key role in the stroma production. Whereas in normal colon TGF-β serves as a suppressor of cell proliferation and may induce apoptosis, an abnormal function of this pathway may contribute to the initiation and progression of cancer.[20,21,22] SMAD proteins are signal transducers in the TGF-β pathway and are essential for the growth suppression function of TGF-β. They act as tumor suppressor molecules of which mutation, deletion and silencing is associated with many types of cancer. To support this, SMAD4 located on chromosome 18q21.1. was found functionally inactivated in 30% of the invasive and metastatic colorectal carcinoma.[23-26]

A similar study as presented in chapter 5 was performed but now focused on stage I-II colon patients in **chapter 6.** To elucidate the molecular basis of this morphological phenomenon, immunohistochemical staining was applied to stain markers involved in pathways related to stromal production and epithelial-to-mesenchymal transition (EMT) such as; β-catenin, TGF-β-R2 and SMAD4. This combined approach showed that SMAD4 further substantiates the prognostic value of the presence of abundant stroma in the primary tumor and identifies for a subgroup of patients with worse survival. In this study of 135 analyzed patients 34 (25.2%) were stroma-high and 101 (74.8%) stroma-low. A significant difference in survival time was observed between the two groups with stroma-high patients showing a worse survival (OS $p<0.001$, HZ 2.73; DFS $p=0.001$, HZ 2.43). A high-risk group could be identified showing stroma-high and abrogation of SMAD4 (OS $p=0.008$, DFS $p=0.005$).

Notably twelve out of 14 (85.7%) patients with stroma-high and SMAD4 abrogation died within 3 years. In a logistic regression analysis stroma and SMAD4 were found to be strongly related (HZ 5.42, CI 2.13-13.82).

In chapter 5 and 6 it was shown that a powerful prognostic parameter could be established using conventional H&E stained slides. The estimation of the tumor-stroma ratio in combination with immunohistochemical staining for SMAD4 abrogation results in an independent prognostic parameter for more confident prediction of clinical outcome.

## Clinical applications and future perspectives

### 1. Detection of micrometastases in lymph nodes

The detection of micrometastases in lymph nodes to which the primary tumor drains has been pursued over the past years.[27] In this thesis we present an optimized protocol for the detection of micrometastases using immunohistochemistry (IHC) and automated microscopy. For the applicability of this method in a clinical setting sectioning and staining of all resected lymph nodes is too labor intensive. However, for the analysis of sentinel lymph nodes, which most of the time involves one to three nodes, serial sectioning is highly recommended by the American ADASP (Association of Directors of Anatomic and Surgical Pathology) and practically feasible.

In a recent paper by Bilchik et al. a first prospective evaluation of the prognostic impact of micrometastases in colorectal cancer using IHC and quantitative RT-PCR was published. Upstaging of 8% of the patients from node-negative (pN0) to node-positive (pN1) was reported. These results indicate that the detection of micrometastases may be clinically relevant and improve the selection of patients for adjuvant chemotherapy. Also a new group of patients may exist with negative lymph nodes by cumulative detection methods (IHC and qRT-PCR) who are likely to be cured by surgery alone.[28]

### 2. Detection of tumor cells in bone marrow and peripheral blood

Within the DISMAL project a European Community's Sixth Framework program, several groups in Europe focus on the standardization of the detection of tumor cells in bone marrow. For breast cancer, a pooled analysis from nine studies involving 4703 patients with stage I, II, or III showed that the presence of micrometastasis in the bone marrow at the time of diagnosis is associated with a poor prognosis.[8] For colorectal cancer the presence of DTC's in BM has been described as an independent prognostic factor but needs to be confirmed in a larger series. [15, 29, 29, 30] Within the LUMC this parameter is currently being evaluated on a large set of (n=300) patients.

As for breast cancer many papers have been published which uniformly support the value of the detection of DTC in BM as a prognostic parameter, but till now this technique is not applied in a clinical setting. This can be due to practicality since the method is labor intensive, or due to lack of an uniform protocol since tests are being performed using RT-PCR or IHC with different markers as CEA and CK20 and cytokeratin specific antibodies such as A45B/B3 and AE1/AE3.

Within the scope of the EU-DISMAL consortium, patient data from different international institutes are being pooled and analyzed to establish one uniform protocol which can be applied in a clinical setting. In the LUMC several new molecular techniques are available as genomic and expression profiling and proteomics, which will also be evaluated within this program.

The analysis of BM-positive versus BM-negative patients offered a high-risk marker for the presence of DTC's in BM. Although the analyzed set of patients is very limited, data looks promising and should be further evaluated within this consortium on a larger patient series.

Since the introduction of the Veridex system (Immunicon Corp.), a system to enrich and detect circulating tumor cells from the peripheral blood, much progress has been made in this field (for more details see also introduction). This system offers the possibility to select and to monitor patients during treatment for which BM sampling is not suited. The presence of more than 3 cells per 7 ml of peripheral blood before treatment has been found to be an independent predictor of progression-free survival and overall survival in patients with metastatic breast cancer.[9]

The recently published technology for the isolation of cells from the peripheral blood using microchip technology by Nagrath et al. offers a new tool for the identification and measurement of CTC's in patients with cancer.[31] The detection of a high number of tumor cells is reported with a range of 5 to 1,281 tumor cells per ml blood of metastatic cancer patients. This would imply at least 30,000 to approx. 6 million cells in the human blood circulation at any given moment which seems to be rather high.

Although the described new technologies are promising and generate good digital images, visual confirmation using microscopy of detected tumor cells remains the most convincing method although parallel studies should be performed.

**3. Determination of the intra-tumor stroma percentage**

In this thesis a new parameter for the identification of high-risk, stage II, colon cancer patients is presented. The percentage of intra-tumor stroma, determined on H&E stained slides from the primary tumor, was found to be a parameter to select for patients with worse outcome of disease. In combination with abrogation of the tumor suppressor gene SMAD4 this parameter selects for a specific group of patients with bad outcome and death due to disease within 3 years. Based on this parameter patients can be selected for adjuvant therapy, but obviously it is not known yet, if this subgroup of patients with worse survival would also benefit from therapy.

For confirmation of the results obtained in this study, the study of the QUASAR Collaborative Group would be unique. This study consists of patients stage II and III, for which the stage II group is partly composed of a treated and an untreated group.

Future research will focus on the molecular background of the presence of abundant stroma within the primary tumor. Published data point towards the involvement of the TGF-β pathway in the tumor-stroma process. [24, 31-38]

For separate analysis of the tumor and stroma components, lasercapture techniques can be applied in combination with array-CGH or expression-array. A drawback of this method however is the high amount of DNA and RNA necessary. MS-imaging (mass spectrometric imaging) by MALDI, which is based on the analysis of tissue samples using mass spectrometry offers the possibility to analyze different components of the tumor for novel protein detection. MS-imaging is leaving the tissue intact for visual evaluation and gives the investigator the opportunity to select specific areas of interest using simple H&E sections. This method is used to measure the characteristics of individual molecules, using a mass spectrometer which converts them to ions so that they can be moved about and manipulated by external electric and magnetic fields. MS imaging is expected to measure the protein distribution in different tissue compartments and may result in identify-

ing key elements involved in the tumor-stroma interaction. Serum proteomics using MALDI-TOF mass spectrometry only, has already resulted in different MS spectra for colon cancer patients in comparison to controls.[39]

The emphasis of this thesis has been to optimize prognostication of patients with colorectal cancer especially for stage II patients who would benefit from additional therapy. The studies presented focused on morphological cell based parameters. However, research in the field of expression array and proteomics is progressing. As a result of this a set of genes has been found that allows for the identification of colon cancer stage II patients with worse outcome of disease. As to be expected, the molecular effort in the field of colorectal cancer will finally result in reliable parameters that correlate with prognosis and outcome of therapy. As serendipity observation in the set-up for one of these techniques we found the stroma parameter as a reproducible and simple prognostic parameter pointing towards important differences in biology.

It is remarkable that a simple cell based parameter (tumor-stroma ratio) using conventional microscopy, with at hardly any additional costs, can give such good predictions. This parameter seems not to be limited to CRC only, recently we also evaluated this parameter as prognostic factor for esophageal and breast cancer, and observed that for the last one it is even of predictive value for systemic therapy. Obviously confirmation of this parameter is needed, for example the QUASAR study would be ideal. If confirmed, further validation of this parameter is needed, which should lead to a prospective randomized trial of stage II colorectal cancer patients.

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# Chapter 8

Nederlandse samenvatting

Dikke darmkanker is wereldwijd de vierde meest voorkomende maligniteit na borstkanker, prostaat en longkanker. Alleen al in Nederland overleden in 2004 2.260 mannen en 2.175 vrouwen ten gevolge van deze ziekte. De incidentie van dikke darmkanker in Nederland is hoog vergeleken bij andere landen. Het aantal nieuwe patiënten per jaar is 9.898 en dit aantal zal blijven toenemen door vergrijzing van de huidige populatie. Ten aanzien van de periode van 2005-2025 wordt verwacht dat het aantal nieuwe patiënten stijgt met 41.7%.

Om deze reden is er begonnen met een bevolkingsonderzoek waarbij mensen boven een bepaalde leeftijd, zonder symptomen, worden onderzocht. De Fecale Occulte Bloed Test (FOBT) toont spoortjes bloed aan in de ontlasting wat kan duiden op de aanwezigheid van een darmtumor. Indien de test elke 2 jaar wordt uitgevoerd vermindert vroegtijdig onderzoek met behulp van de FOBT het risico op overlijden ten gevolge van darmkanker met 15-20%.

Voor patiënten met een tumor is de aanwezigheid van lymfeklier metastasen in veel gevallen van kanker een zeer belangrijke prognostische parameter, die bepalend kan zijn voor de therapie, zo ook voor darmkanker. De "American Joint Committee on Cancer (AJCC)" werkt sinds 1959 aan een uniform classificatiesysteem voor de stagering van kanker. Dit internationaal geaccepteerde systeem voorziet in een nauwkeurige beschrijving van de verschillende typen tumoren met als doel een universeel systeem te bieden dat kan worden gebruikt voor de beschrijvende ziekteleer, om patiënten groepen te vergelijken en therapie te bepalen, patiënten te selecteren voor klinische trials, resultaten van klinische trials te analyseren en de prognose met de patiënt te bespreken.

De algemeen geaccepteerde prognostische factoren voor dikke darmkanker zijn lokalisatie van de tumor, grootte (T), de aanwezigheid van lymfeklier metastasen (N) en metastasen op afstand (M), allen deel uitmakend van het klassieke TNM classificatiesysteem.

Patiënten met negatieve lymfeklieren (TNM stadium I en II) krijgen doorgaans geen aanvullende chemotherapie of radiotherapie. Echter, vijfentwintig procent van alle stadium II patiënten vertoont terugkeer van de ziekte. Het geven van chemotherapie (fluorouracil en foliumzuur) aan alle stadium II patiënten geeft echter maar een behandelingswinst van 3.6%, zoals onlangs is gerapporteerd in een publicatie van de "QUASAR Collaborative Group" en is onder de grens van 5% die als minimum wordt geaccepteerd. Dit heeft er toe geleid dat chemotherapie niet als standaardtherapie aan stadium II patiënten wordt gegeven, maar alleen aan patiënten met een verhoogd risico op terugkeer van de ziekte (recidief), zoals aan patiënten met een T4 tumorstadium, doorgroei van de tumor in de bloedvaten, een laag aantal verwijderde lymfeklieren (N<12) en perforatie of obstructie. Aannemende dat de 5-jaars mortaliteit zonder chemotherapie 20% is, zou behandeling van deze groep resulteren in een absolute winst van 5.4% voor hoogrisico patiënten in vergelijking tot 3.6% voor de totale stadium II groep.

Hoewel er verschillende opties zijn voor het geven van aanvullende therapie is de klinische vraag die voor ligt evident: hoe kan een behandeling gekozen worden die het best past bij de individuele patiënt?

Op dit moment staat de prognostische waarde van de huidige TNM classificatie ter discussie. Door de hedendaagse vroege detectie van tumoren is de grootte van de

tumor minder belangrijk geworden. De vraag is dan ook of het niet méér relevant is om parameters die de biologie van de tumor reflecteren te gebruiken als minder subjectieve, onafhankelijke parameter voor prognose. De tumorbiologie kan tevens worden gebruikt als voorspellende parameter ten aanzien van de respons op de behandeling.

In dit proefschrift zijn microscopische, op celniveau gebaseerde, prognostische parameters beschreven om patiënten te selecteren voor aanvullende therapie.
Automatische microscopie is toegepast om de analyse van lymfeklieren op het voorkomen van tumorcellen, de belangrijkste parameter van de TNM classificatie, te verbeteren. De tumor-stroma verhouding van de primaire tumor is als nieuwe parameter geintroduceerd. Er is ook onderzoek gedaan naar genomische veranderingen in primair tumorweefsel die correleren met de aanwezigheid van gedissemineerde tumorcellen in het beenmerg.

Een globaal overzicht over dikke darmkanker wordt gegeven in **hoofdstuk 1**. In dit hoofdstuk zijn parameters besproken die momenteel worden gebruikt in de routine pathologie voor het selecteren van patiënten met een verhoogd risico op terugkeer van de ziekte. Verder zijn nieuwe biologische en moleculaire prognostische markers besproken en hun relatie tot het huidige TNM classificatiesysteem.

Biologische parameters zoals de aanwezigheid van micrometastasen (kleine groepjes tumorcellen >0.2 mm en <2 mm) in lymfeklieren, gedissemineerde tumorcellen in beenmerg (DTC's) en circulerende tumorcellen (CTC's) in perifeer bloed zijn van prognostisch belang en geven inzicht in het proces van disseminatie en metastasering zoals beschreven in hoofstuk 6 van dit proefschrift. Echter, het vinden van deze vaak zeldzaam voorkomende cellen wordt bemoeilijkt door hun lage frequentie. Standaard microscopische beoordeling van meerdere lymfekliercoupes of preparaten, gemaakt van beenmerg of perifeer bloed, is een tijdrovende en moeilijk te reproduceren taak. Automatische microscopie kan deze taak in belangrijke mate ondersteunen. In **hoofdstuk 2** worden de mogelijkheden voor automatische analyse van lymfeklieren, beenmerg en bloed met behulp van automatische systemen besproken. Hierbij wordt software gebruikt om de cellen te identificeren op basis van hun immuunhistochemische of immuuncytochemische kleurresultaat. Een patholoog beoordeelt de geselecteerde objecten en bepaalt de uiteindelijke uitslag. In dit hoofdstuk worden de mogelijkheden besproken van de huidige commercieel verkrijgbare apparatuur en de toepasbaarheid van deze apparatuur voor routinediagnostiek.

In **hoofdstuk 3** wordt automatische microscopie toegepast voor het vinden van micrometastasen in de lymfeklieren van stadium II patiënten om patiënten te identificeren met een hoog risico op terugkeer van ziekte. Een soortgelijke studie is eerder uitgevoerd door Liefers et al met behulp van RT-PCR (reverse transcriptase-polymerase chain reaction) voor CEA (carcino-embryonaal antigeen) in histologisch negatieve lymfeklieren. De 5-jaar overleving voor de groep patiënten met micrometastasen in de lymfeklieren was 50% ten opzichte van 91% voor de groep zonder micrometastasen (p=0.02).

Dezelfde patiëntengroep werd nogmaals onderzocht, waarbij een flatbed-scanner is gebruikt om paraffinecoupes van het lymfeklierweefsel te analyseren. Paraffineblokken werden serieel gesneden en immuunhistochemisch gekleurd met behulp van antilichamen tegen het cytokeratine van de cellen. Van elke lymfeklier zijn 10 glaasjes gemaakt met elk 5 coupes van 10 verschillende snij-niveaus. De resultaten uit deze studie laten zien dat, door het aansnijden van meerdere niveaus van de lymfeklier, en immuunhistochemische kleuring voor cytokeratine, toch micrometastasen worden gevonden bij alle (100%) klier-negatieve patiënten met een recidief. Bij patiënten zonder recidief werden in 43% micrometastasen in de lymfeklieren gevonden. Deze resultaten zijn vergelijkbaar met die van Liefers et al. wat voor deze patiënten een verandering in TNM classificatie van stadium II naar stadium III en dus aanvullende therapie kan betekenen.
Met behulp van flatbed-scanning is het mogelijk meer microscopische preparaten te analyseren dan in de routinepathologie waardoor een grotere gevoeligheid wordt bereikt. De automatische analyse van lymfekliercoupes is inmiddels geïmplementeerd in het software programma van het ARIOL systeem (Applied Imaging, Genetix). Dit resulteerde in een vergelijkbare gevoeligheid, maar in een meer gebruikersvriendelijke methode die geschikt is voor routinematige toepassingen.
In een volgende studie werd onderzocht hoeveel snij-niveaus noodzakelijk zijn om alle patiënten met een recidief op te sporen. Uit deze studie is gebleken dat de analyse van 4 snij-niveaus met een tussenstap van 200 µm voldoende is als compromis tussen gevoeligheid en praktische toepasbaarheid. Men zou kunnen overwegen om deze techniek te implementeren in de routine pathologie. Echter, gezien het aantal chirurgisch verwijderde lymfeklieren in dikke darmkanker per patiënt vaak 20 is of meer, lijkt het aangewezen deze aanpak te reserveren voor de analyse van poortwachterklieren.

De aanwezigheid van tumorcellen in het beenmerg (BM) van patiënten met kanker wordt geassocieerd met een slechte prognose; dit geldt met name voor patiënten met borstkanker. Om deze parameter ook te evalueren voor dikke darmkanker is het LUMC vanaf het jaar 2000 begonnen met het verzamelen van beenmerg van patiënten met CRC. Een gestandaardiseerde methode werd ontwikkeld die bestond uit de aspiratie van 5 ml BM van beide cristae, de preparatie van glaasjes met 2 miljoen cellen en antilichaamkleuring voor het cytokeratine van de cellen, gevolgd door automatische microscopische analyse van de verkregen glaasjes. Tot op heden zijn ongeveer 300 patiënten geanalyseerd waarin in 15% van deze patiënten tumorcellen in het beenmerg zijn gevonden.

**Hoofdstuk 4** beschrijft het gebruik van array-CGH om genomische afwijkingen te vinden in primaire tumoren van BM-positieve patiënten in vergelijking met BM-negatieve patiënten die, op basis van tumor locatie en TNM classificatie, overeen kwamen. Het doel van deze studie was een specifiek genomisch profiel te identificeren dat correleert met het ontstaan van micrometastasen in beenmerg. Vergeleken met de BM-negatieve groep werd een hoger aantal genomische veranderingen gevonden in de BM-positieve groep welke tevens bevestigd zijn met

FISH (fluorescentie in situ hybridisatie). Specifieke chromosoomgebieden als 6p (p21.1), 9p (p11.2-p13.3), 12q (q13) en 16 en 19 (hele chromosoom) waren vaker in verhoogd kopie-aantal aanwezig in de BM-positieve groep, evenals het verlies van chromosoom 11q (q22.3-q25) en 15q (q11.2-q12 en q14-q21). In dit hoofdstuk is een interessante set markers geïdentificeerd die weliswaar niet uitsluitend maar voornamelijk werd gevonden in patiënten met gedissemineerde tumorcellen in het beenmerg. Verder onderzoek is noodzakelijk om de waarde van deze markers vast te stellen.

Indien array-CGH wordt toegepast voor onderzoek van tumorweefsel, is het gebruikelijk om die delen van de tumor te selecteren waarin de tumor het grootste aandeel vormt. Vermenging met stroma of ontstekingsweefsel kan de amplificaties (toename) en deleties (afname) van bepaalde stukjes DNA maskeren. Tijdens een nieuw op te zetten onderzoek was het opvallend dat voor een bepaalde set patiënten, met een slechte prognose, de hoeveelheid tumormateriaal minder was dan 50% en dat de primaire tumor voornamelijk uit stroma bestond. Omdat deze samples niet voldeden aan de criteria voor een betrouwbare array-CGH, werden ze beschouwd als niet geschikt voor analyse. Tumoren met een goede prognose vertoonden echter het tegenovergestelde; veel tumor en weinig stroma. Deze observatie heeft geleid tot de evaluatie van deze parameter in een grotere patiëntenserie waarvan de resultaten zijn gepresenteerd in **hoofdstuk 5**. In een retrospectieve studie van 122 patiënten (stadium I-III) met tenminste 10 jaar follow-up, werden 33 patiënten (27%) als stroma-hoog en 89 (73%) als stroma-laag geclassificeerd. De vijf-jaar overleving van deze patiënten was respectievelijk 15.2% en 73.0% en de ziektevrije overleving 12.1% en 67.4%, waarbij stroma-hoog patiënten het slechter deden (OS $p<0.0001$, HZ 3.73; DFS $p<0.0001$, HZ 4.18). In een multivariaat Cox regressie-analyse bleek de hoeveelheid stroma een onafhankelijke parameter te zijn in relatie tot het stadium, of tot de tumorstatus en lymfeklierstatus (OS $p<0.001$, DFS $p<0.001$)
De "Transforming Growth Factor-β" (TGF-β) pathway is betrokken bij de stromaproductie. Terwijl in het normale darmweefsel TGF-β de functie heeft van suppressor van celproliferatie en apoptose kan induceren, kan een afwijkende functie van deze pathway bijdragen tot de initiatie en progressie van kanker. SMAD eiwitten zijn signaaltransductiemoleculen van de TGF-β pathway en essentieel voor de groei-suppressiefunctie van TGF-β. Ze zijn werkzaam als tumorsuppressor-moleculen waarvan de mutatie, deletie en silencing zijn geassocieerd met verschillende typen kanker. SMAD4, waarvan het gen zich bevindt op chromosoom 18q21.1, is afgeschakeld in 30% van de invasieve en gemetastaseerde dikke darmtumoren.

Om de morfologische stroma-tumor verhouding moleculair te onderbouwen is in **hoofdstuk 6** immuunhistochemie toegepast voor markers die betrokken zijn bij de stromaproductie en de epitheliale-mesenchymale transitie (EMT), namelijk β-catenine, TGF-β-R2 en SMAD4. Een vergelijkbare studie als gepresenteerd in hoofdstuk 5 werd uitgevoerd, maar nu gericht op stadium I en II patiënten. In deze studie is gevonden dat SMAD4 de prognostische waarde van de stroma parameter verder kan versterken. In deze studie

zijn 135 patiënten geanalyseerd waarvan 34 (25.2%) stroma-hoog en 101 (74.8%) stroma-laag. Er was een significant verschil in overlevingstijd tussen beide groepen, waarbij stroma-hoog patiënten een slechte overleving hadden (OS p<0.001, HZ 2.73; DFS p=0.001, HZ 2.43). Een hoog-risico groep patiënten met een hoog stroma percentage en afschakeling van SMAD4 (OS p=0.008, DFS p=0.005) kon worden geïdentificeerd waarvan 12 van in totaal 14 patiënten (85.7%) overleden bleken te zijn binnen 3 jaar. De regressie-analyse laat een duidelijke relatie zien tussen stroma en SMAD4 (HZ 5.42, CI 2.13-13.82).
In hoofdstuk 5 en 6 is aangetoond dat routine H&E histologische coupes een krachtige nieuwe parameter leveren. De bepaling van de tumor-stroma ratio in combinatie met immuuncytochemische kleuring voor SMAD4 resulteert in een onafhankelijke parameter voor een meer betrouwbare voorspelling van het ziektebeloop in dikke darmkanker.

In dit proefschrift zijn prognostische parameters gepresenteerd om patiënten met dikke darmkanker te selecteren, die baat kunnen hebben bij aanvullende behandeling. Deze studies zijn gebaseerd op microscopische, op celniveau gebaseerde parameters. Echter, men moet ook vaststellen dat met technieken als expressieprofiling en proteomics veel voortgang is geboekt met betrekking tot het vinden van nieuwe prognose gerelateerde parameters. Zo is er een set genen gevonden op grond waarvan patiënten geselecteerd kunnen worden met een slecht ziektebeloop.
Deze moleculaire ontwikkelingen zullen waarschijnlijk uiteindelijk resulteren in betrouwbare parameters die correleren met prognose en therapie. Maar het is opvallend dat een eenvoudige histologische parameter (tumor-stroma ratio), waarvoor slechts conventionele microscopie vereist is, zo'n krachtige voorspellende waarde heeft.
Verdere validatie van de tumor-stroma ratio in een multicenter studie is echter noodzakelijk. Dit zou in korte tijd moeten leiden tot een gerandomiseerde trial van stadium II patiënten met dikke darmkanker.

# Curriculum Vitae

Wilma Mesker (12 juni 1963, Leiden) behaalde het diploma MAVO-B aan de Willibrord MAVO te Noordwijk in 1979 en haar HBO diploma (richting histo-cyto-pathologie) aan de Hogere Laboratoriumschool te Leiden in 1982. In het laatste jaar van haar opleiding heeft ze haar stageperiode vervuld bij de afdeling Pathologie van Prof. R. Donner aan de Vrije Universiteit te Amsterdam.
Na haar opleiding was ze twee jaar werkzaam bij de afdelingen Pathologie en Cytologie van Prof. J.F. Delemarre en Dr. P. van Heerde in het Antoni van Leeuwenhoekhuis te Amsterdam .
Sinds 1984 is zij werkzaam op de afdeling Cytochemie en Cytometrie (Prof. Dr. J.S. Ploem) nu onderdeel van de afdeling Moleculaire Celbiologie (Prof. Dr. H.J. Tanke) van het Leids Universitair Medisch Centrum.
In 1988 haalde zij de Amerikaanse graad (CTIAC) in de Cytologie aan de International Academy of Cytology in Brussel.
Haar onderzoek op de afdeling Moleculaire Celbiologie betrof de automatische microscopie, beeldbewerking en klinische toepassingen hiervan zoals automatische analyse van baarmoederhalsuitstrijkjes en het vroegtijdig detecteren van CMV reactivatie bij niertransplantatie patiënten. Ook werd lange tijd samengewerkt met de afdeling Verloskunde (Dr. S. A. Scherjon, Prof. Dr. H.H.H. Kanhai) betreffende de detectie van circulerende foetale cellen in de maternale circulatie voor het vroegtijdig opsporen van chromosomale afwijkingen.
Sinds 2000 is er een hechte samenwerking met de afdeling Heelkunde (Prof. Dr. R.A.E.M. Tollenaar) op het gebied van translationeel onderzoek van colorectale tumoren waarbinnen de mogelijkheid voor deze promotie is ontstaan. Sinds januari 2008 is zij tevens parttime als onderzoeker werkzaam bij deze afdeling.

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Figures Chapters 3, 4 and 6

# Chapter 3

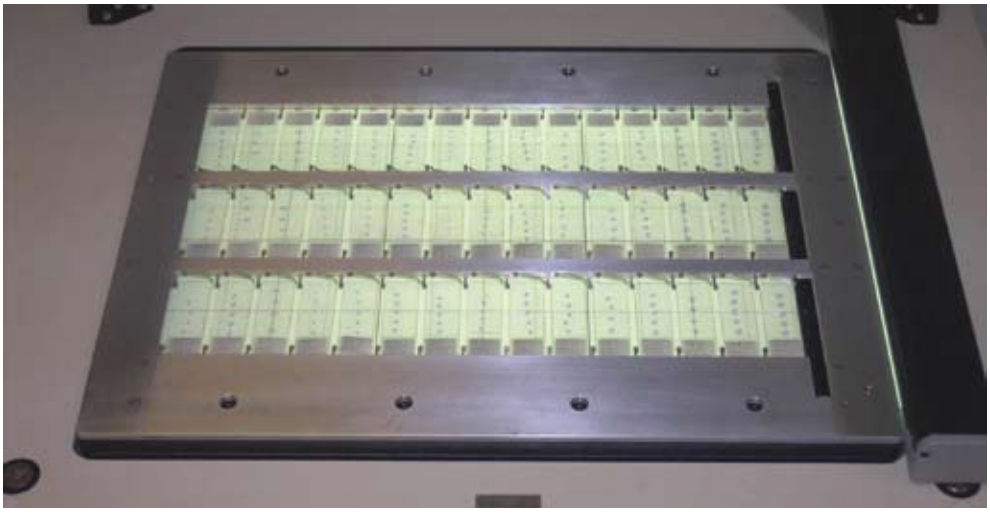

*Fig. 1* A3-size mold to hold $15 \times 3 = 45$ slides on the AGFA XY15 scanner. Each slide may contain several tissue sections.

A


B

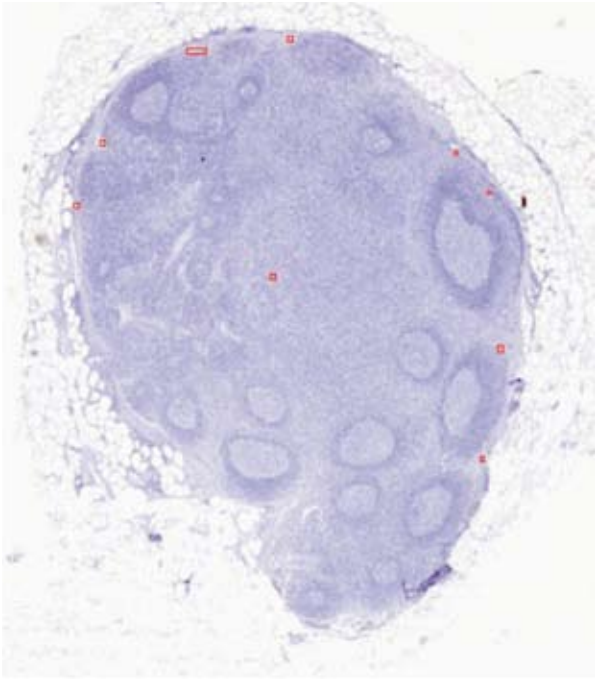

*Fig. 2* *A*, gallery with images of candidate occult cells detected in a lymph node section of a patient with colorectal carcinoma. Selection of candidate cells is based on immunoperoxidase (*brown*) staining for CK. Counterstaining is performed with hematoxylin (*blue*). *B*, overview of the lymph node with markers displaying the location of the candidate cells.

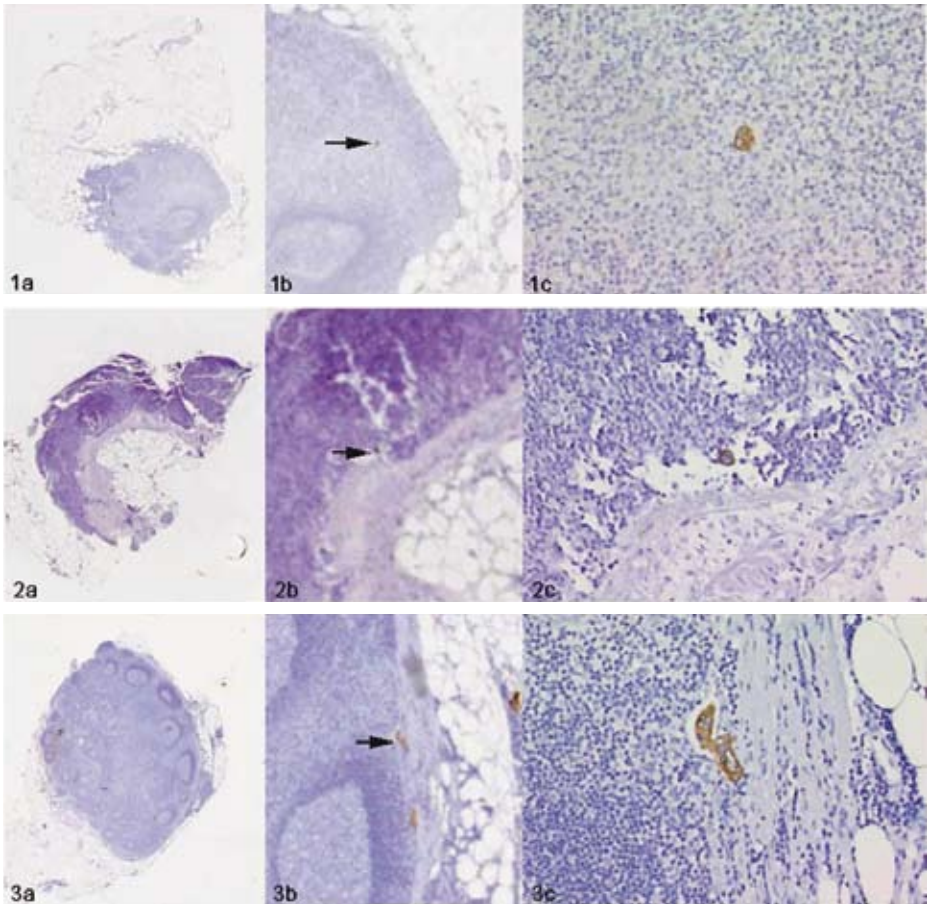


*Fig. 3* *a*, examples of images recorded by the flatbed scanner at 5600 dpi. Sections of lymph nodes from patients with colorectal carcinoma. Cells are positively stained for CK and counterstained using hematoxylin. *b*, zoom function of recorded image; *arrow*, a small group (*3b*) or single $CK^+$ cells (*1b*, *2b*). *c*, conventional microscope image of the same cells. Recorded with color CCD camera using ×16 objective.

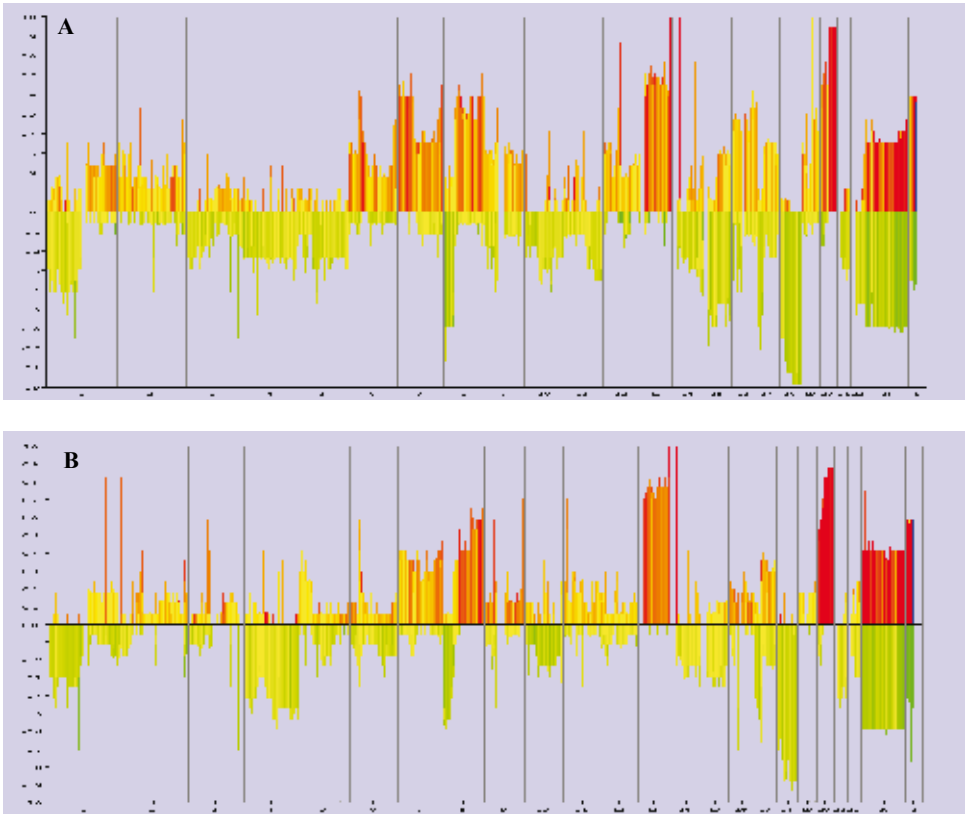


Figure 2.
Overall frequency of DNA copy number alterations by array-based CGH. Frequency analysis (y-axis) measured as a fraction of cases gained or lost over the 3500 BAC clones.
Data are presented ordered by chromosomal map position of the clones (x-axis).
Lower bars represent losses or deletions for all clones, and the upper bars represent gains or amplifications. Red: amplification, green: deletion, yellow: below the threshold of 25%.
Data have been generated by VAMP software using the FrAGL (Frequency of Amplicon, Gain and Loss) option. a. BM-positive patients, b. BM-negative patients.

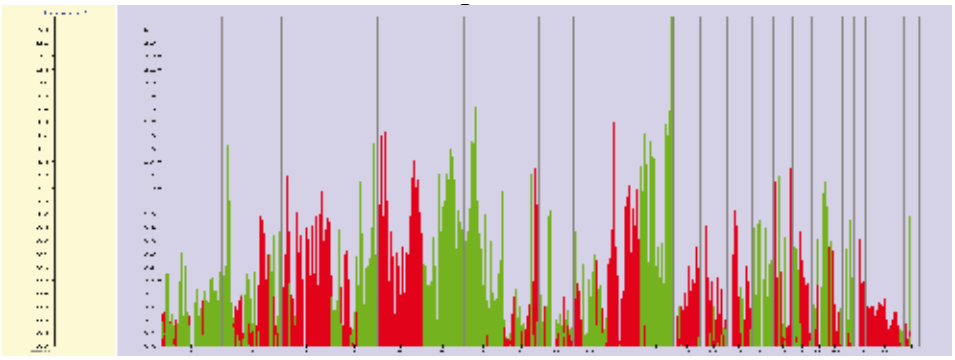

Figure 3.
Differential analysis of DNA copy number alterations by array-based CGH of BM-positive patients compared to BM-negative patients. Frequency analysis (y-axis) measured as a fraction of cases gained or lost over the 3500 BAC clones.
Data are presented ordered by chromosomal map position of the clones (x-axis). Red: amplification, green: deletion.
Data have been generated by VAMP software using the FrAGL (Frequency of Amplicon, Gain and Loss) option.

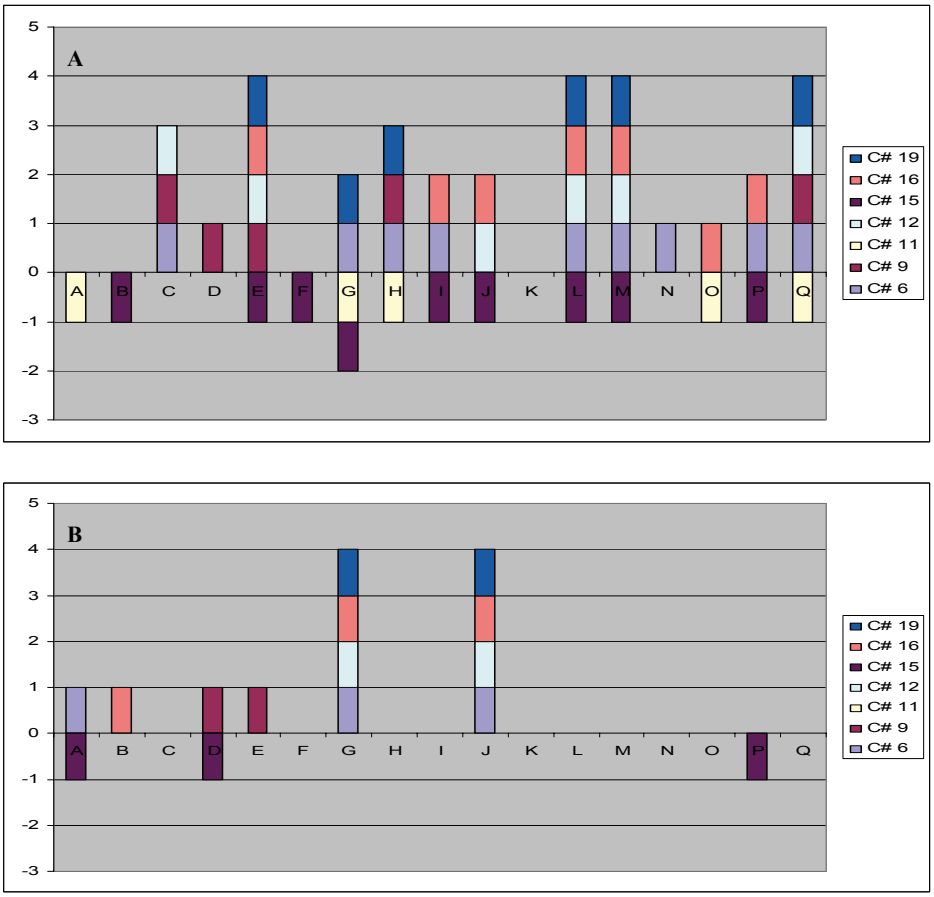


Figure 4.

DNA copy number alterations by array-based CGH of BM-positive patients compared to BM-negative patients estimated by differential analysis of FrAGL supported by VAMP software for the most frequent gained chromosomes 6, 9, 12, 16 and 19 and losses of 11q and 15q.
(X-axis: patients are displayed as numbers A-Q, Y-axis: number of alterations)
a. BM-positive patients (analyzed liver samples have the numbers: B, J, M, P), b. BM-negative patients (analyzed liver samples have the numbers: D, K, L, O).

# Chapter 6

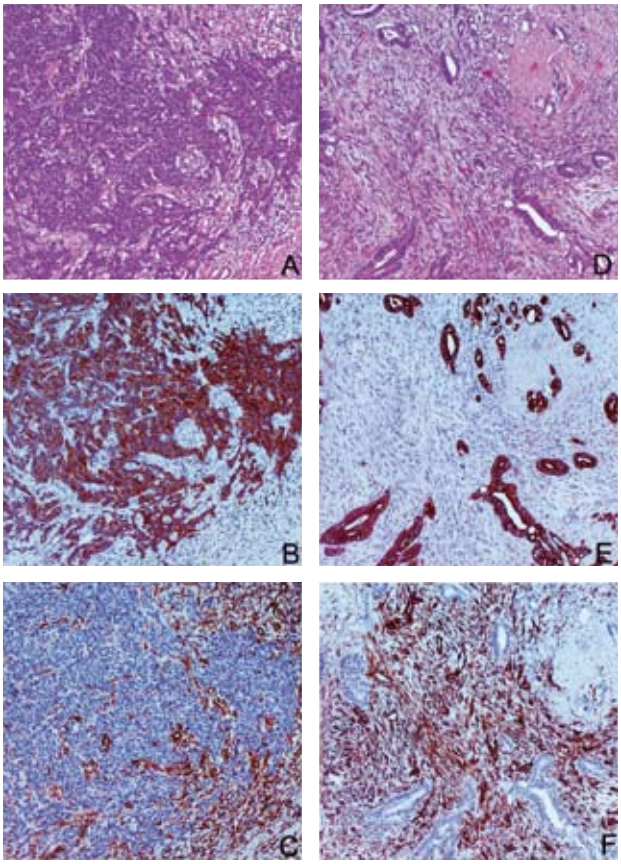


Fig. 1. H&E stained 5 μm paraffin sections of primary colon tumors. Carcinoma percentage estimated as 80% in a patient with long OS/DFS: (a) H&E staining; (b) cytokeratin staining for carcinoma cells; (c) vimentin staining of stromal compartments. Carcinoma percentage estimated as more than 30% in patient with short OS/DFS: (d) H&E staining; (e) cytokeratin staining for carcinoma cells; (f) vimentin staining of stromal compartment.