Improving diagnostic, prognostic and predictive biomarkers in colorectal cancer: the role of proteomics and stromatogenesis

Huijbers, A.

Improving Diagnostic, Prognostic and Predictive Biomarkers in Colorectal Cancer

The role of Proteomics and Stromatogenesis

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Colophon:
This thesis was financially supported by: Logistikos and Rotary Lisse-Bollenstreek.
Cover design: Chantal Vroom
Printing: Optima Grafische Communicatie

ISBN: 978-94-6361-655-3

Improving Diagnostic, Prognostic and Predictive Biomarkers in Colorectal Cancer
The role of Proteomics and Stromatogenesis

Proefschrift

ter verkrijging van
de graad van doctor aan de Universiteit Leiden,
op gezag van rector magnificus prof.dr.ir. H. Bijl,
volgens besluit van het college van promoties
te verdedigen op donderdag 2 juni klokke 15:00 uur

door

Anouck Huijbers
geboren te Alphen aan den Rijn

PROMOTIECOMMISSIE

Promotor: Prof. Dr. R.A.E.M. Tollenaar

Co-Promotor: Dr. W.E. Mesker

Leden Promotiecommissie:
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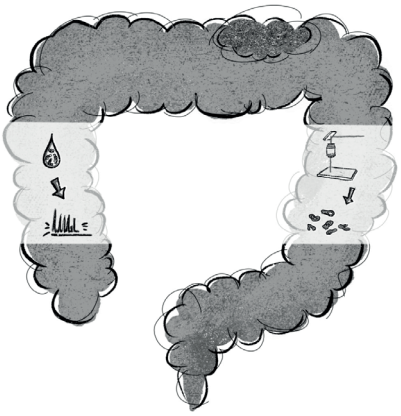

I

Introduction and Outline

INTRODUCTION

Colorectal cancer is one of the most common diagnosed cancers, with incidence rates ranking second in women and third in men. Moreover, it is the third and fourth leading cause of cancer-related deaths in women and men, respectively [1].

Most colorectal cancers originate from normal colonic mucosa through an adenoma-carcinoma sequence. The prognosis of colorectal cancer mainly depends on the tumor stage. Therefore, early diagnosis is of great importance to reduce disease-related mortality [2, 3].

Despite histologic tumor staging, stage-independent outcome variability occurs, which probably reflects tumoral molecular heterogeneity. Even patients with early stage colorectal cancer may show disease relapse and cancer progression despite initial surgical management. This indicates a need for biomarkers that contribute to risk stratification of colorectal cancer beyond conventional clinicopathological staging [4].

This thesis analyses the pathologic and molecular characterizations of colorectal cancer. It thereby focuses on the role of biomarkers to improve disease specific survival. A biomarker – or biological marker – is a measurable indicator that reflects normal biological processes, pathogenic processes or responses to an exposure or intervention [5]. Biomarkers are often defined in accordance with how they are applied. This research focuses on three particular subtypes of biomarkers: diagnostic, prognostic and predictive biomarkers and their role on improving colorectal cancer outcome.

First, a *diagnostic* biomarker detects or confirms the presence of a disease or medical condition of interest [6]. Second, a *prognostic* biomarker is used to identify the likelihood of a clinical event, disease recurrence, or disease progression in patients with a disease or medical condition of interest [6]. And third a *predictive* biomarker is present or changes to indicate that an individual or group of individuals is more likely to experience a favourable or unfavourable effect from the exposure to a medical product or environmental agent [6].

This doctoral thesis consists of two parts. In the first part, the research analyses the role of proteomics as a *diagnostic* biomarker for early colorectal cancer detection. This research is relevant because through its use as a diagnostic biomarker, proteomics may improve screening applications.

The second part of this research examines the role of stromatogenesis as a *prognostic* and *predictive* biomarker. This part of the research promotes the development and selection of personalised therapies based on the pathological and molecular findings that result from analysing stroma tissue.

This research as a whole thereby offers new insights into colorectal cancer diagnostic biomarkers that could ultimately help improve and simplify early detection and prognostic and predictive biomarkers that define the optimal personalized treatment for patients in routine clinical practice; thereby improving their survival.

OUTLINE

Part 1 Proteomics as a Diagnostic Biomarker

Early diagnosis of cancer is of pivotal importance to reduce disease-related mortality. Therefore, non-invasive screening methods can offer a vital improvement for colorectal cancer survival. However, existing screening protocols have limited sensitivity and specificity [7-10].

The use of serum biomarkers to distinguish cancer patients from healthy persons may be a tool to improve screening programs. Serum is an ideal sample type for early detection markers since samples can be obtained in a straightforward, standardised manner at minimal cost, minimal risk and, most importantly, in a less-invasive manner compared to existing detection methods, such as colonoscopy [11]. i. Mass spectrometry-based proteomics is widely applied for mapping and identifying peptides and proteins in body fluids [12-16].

Chapters 2 and 3 focus on a possible role of proteomic serum biomarkers in screening programmes. Specifically, **chapter 2** provides an overview of and reviews profiling methods that have the potential to be implemented in a clinical setting and national screening programs. **Chapter 3** offers a case-controlled study that identifies proteomic profiles and their potential for colorectal cancer screening. In that study, serum samples were obtained from 126 colorectal cancer patients (CRC) before treatment and 277 healthy control individuals. An additional group of samples from 50 CRC patients and 82 controls was used for validation. Peptide and protein profiles were acquired on a matrix-assisted laser desorption/ionization time-of-flight system and the results were validated on an identical patient set. This resulted in a relatively simple and cheap test that could be promising for improving current CRC screening and reducing the number of necessary colonoscopies.

Part 2 Stromatogenesis as a Prognostic and Predictive Biomarker

Chapters 4-6 focus on stromatogenesis. Stromatogenesis is the formation of new specific types of tumor stroma. Tumor stroma is mainly composed of fibroblasts and extracellular matrix, at sites of active tumor cell invasion. There are many types of stromal formation, such as the usual reactive fibrosis that surrounds benign neoplasms (fibrous capsule), or the formation of avascular connective tissue that fills the gap of a wound (scar tissue). However, this type of stroma in malignant tumors is of a different kind. It facilitates tumor cell invasion and migration. Therefore, tumor-stroma and cancer cell interactions may be key elements in the puzzle of tumor survival, growth, invasion and metastasis [17].

Tumor stroma as a prognostic factor for stage II and III colon cancer patients

The pathologic variables that are commonly used nowadays as indicators of outcome and therapy response can be improved. Therefore, more specific markers are necessary that i) contribute to unravelling the molecular heterogeneity of colon cancer, ii) that discriminate between high- and low-risk groups, and iii) that can possibly even predict therapy response. A prognostic biomarker is the tumor stroma ratio (TSR), which is based on microscopic pathological analysis on conventional hematoxylin an eosin (H&E)-stained paraffin sections. Assessment is fast, cheap and reliable. Previous research by our group demonstrated that the TSR in colon cancer patients is a strong independent prognostic parameter [18, 19]. Patients with a high stroma percentage within the primary tumor have a poor prognosis.

Chapter 4 provides a validation of the TSR based on a large group of 710 patients with stage II and III colon cancer that participated in the VICTOR trial [20, 21]. The VICTOR trial was a randomized clinical trial where patients with stage II and III CRC after complete potential curative treatment were randomized for adjuvant rofecoxib or placebo. In our study, tissue samples from the most invasive part of the tumor were used for analysis of the TSR, using conventional microscopy. We also investigated the possible additional prognostic value of the TSR next to current parameters such as the ASCO high-risk criteria (T4 tumor stage, lymph node yield<10 nodes in the resection specimen, poor tumor differentiation, vascular invasion or perforation of the bowel wall at presentation) and microsatellite instability status that are used in routine pathology reporting.

Bevacizumab for high-risk stroma-high colon cancer patients.

TSR distinguishes between aggressive and non-aggressive tumors. However, it is not yet entirely clear why TSR makes this distinction since the underlying mechanism driving stromatogenesis is still not fully understood. Nevertheless, we do know that tumor

stroma plays an important role in tumor formation and progression [22]. Moreover, one of the factors of tumor progression is angiogenesis, which is facilitated by the tumor stroma. The tumor stroma environment contains multiple different cells including (cancer-associated) fibroblasts, angiogenic vascular cells, and infiltrating immune cells [23]. The prognostic value of TSR has been demonstrated previously [18,19], but its use in therapy selection is a promising new approach to improve TSR-high patients' survival. The stromal environment contributes to tumor angiogenesis, which supplies the oxygen and nutrients needed for tumor growth and progression [24]. Anti-angiogenic therapy, for example with bevacizumab, a monoclonal antibody against vascular endothelial growth factor, can therefore play an important role in treating patients with increased angiogenesis.

Chapter 5 provides our investigation into the added value of bevacizumab to standard chemotherapy for high-risk patients in the QUASAR2 trial. The QUASAR 2 trail was a large phase III randomized trial of adjuvant capecitabine (CAP) ± bevacizumab (BEV) after complete surgical resection of high-risk stage II and stage III colorectal cancer [24]. Tissue samples of the primary tumor of 965 colon cancer patients were analysed for TSR. The study analysed the relation between TSR and the presence of vascular invasion.

Evaluation of the molecular architecture of the tumor associated stroma in colon cancer patients

To effectively address the improvement of survival of colorectal cancer patients, it is of great importance to understand why patients with a high stromal percentage have a poor prognosis, what causes the aggressiveness of high stromal formation, and what pathways are involved in this process. To evaluate the architecture of the tumor-associated stroma tissue, **chapter 6** provides a pilot study that used laser capture microdissection (LCM) that was coupled to broad-scale protein pathway activation mapping, and subsequently reverse phase protein microarrays (RPMA). This technique uses cellular enrichment of specific tissue cells via LCM for tissue biomarker discovery and might contribute to future selection criteria for personalized treatment [15, 16, 26, 27]. It is a high throughput multiplex proteomic platform which can measure hundreds of analytes in a large number of samples with only a small amount of biological material [28-30]. This technique could identify new stromal-based targeted information that offers insights for treatment options because it helps identify activated pathways within the tumor stroma of patients with aggressive colon cancer.

Patients with histologically proven stage II and stage III colon cancer were selected from a LUMC database. TSR was defined and patients were grouped in a stroma-high

or stroma-low group. For this feasibility study, we analysed only evident cases with ≤30% stroma or ≥70% stroma. We performed reverse phase protein microarray that used microdissected material to generate multiplexed pathway profiling for both groups. For this study, we selected 58 proteins and phosphoproteins. The analytes were chosen based on their involvement in key aspects of epithelial mesenchymal transition, extra-cellular matrix composition and remodeling, angiogenesis, inflammation, and transcription. We compared the results in activation/phosphorylation and expression levels of the different analytes between both groups. Furthermore, we did correlation analysis to analyse the possible interactions between different analytes.

Conclusions and Future Perspectives

Finally, **chapter 7** includes a summary of this thesis as well as conclusions and discussion on future perspectives. **Chapter 8** provides a summary in Dutch.

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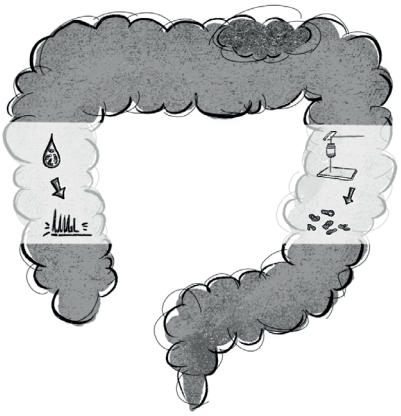

2

Proteomic serum biomarkers and their potential application in cancer screening programs

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International Journal of Molecular Sciences 2010, 11(11), 4175-4193.

ABSTRACT

Early diagnosis of cancer is of pivotal importance to reduce disease-related mortality. There is great need for non-invasive screening methods. Current screening protocols still have limited sensitivity and specificity. The use of serum biomarkers to discriminate cancer patients from healthy persons might provide a chance for improving screening programs. Mass spectrometry based proteomics is widely applied as a technology for mapping and identifying peptides and proteins in body fluids. One commonly used approach in proteomics is peptide and protein profiling. Here we present an overview of profiling methods that have the potential for implementation in a clinical setting and national screening programs.

INTRODUCTION

Population wide screening programs are used to detect early stage cancer to enable early intervention and reduce morbidity and mortality. Ideally screening test have to be highly specific, sensitive, cost-effective and non-invasive. The development of new screening methods has become important due to an increasing incidence, as is the case for colorectal cancer (CRC). In addition, novel screening strategies aim at improved sensitivity and specificity in case of breast cancer. Advanced cancer has a poor survival, whereas when diagnosed at an early stage survival is relatively good [1]. Early detection will identify cancer when it is still localized and curable, preventing not only mortality, but also reducing morbidity and costs [1-5]. The use of serum biomarkers as an indicator of disease in cancer screening programs could provide a promising alternative to existing methods.

A biomarker, or biological marker, is a biomolecule that can be used as an indicator of a disease, based on abnormal presence, absence or changes in genes, RNA, proteins or metabolites. In this manuscript we will discuss protein biomarkers. The ideal biomarker is both highly specific and sensitive. For screening programs the required measurements have to be reliable, robust, fast, and cheap. The material containing the marker(s) should be obtainable in an easy and patient-friendly way. In this respect, body fluids such as serum are suitable sources of biomarkers. Possible applications are (early) detection, prediction of survival and prediction and monitoring of response to therapy. Here we focus on the use of protein biomarkers for early cancer detection.

The translation of the DNA code results in protein expression. In contrast to the genome, the proteome reflects a more dynamic state of the cell [6]. During transformation of a normal cell into a neoplastic cell, distinct changes occurs at the protein level, including altered expression, different protein posttranslational modifications, changes in specific activity and inappropriate localization, all of which may affect cellular function [4;7]. By comparing the protein patterns, i.e. profiles, in serum from patients with cancer with those obtained from healthy individuals, proteins that are the most discriminating can be classified. The resulting protein fingerprint has the potential to identify a person with cancer. Mass spectrometry (MS) has been shown to be a powerful tool in obtaining such protein fingerprints due to its high sensitivity and specificity. In fact, proteomic research has benefitted enormously from developments in MS-technology and has evolved into a new field that is referred to as MS-based proteomics [8]. Whereas proteomics aims for the full identification and quantification of all expressed proteins, profiling strategies usually are applied on sub-sets of the proteome. Importantly, all steps in MS-based profiling methods can be fully automated

allowing high sample throughput and standardization [9]. In finding biomarkers for early cancer detection the content of this review is limited to results obtained from protein profiling efforts.

SCREENING FOR BREAST CANCER AND COLORECTAL CANCER

Breast cancer

Breast cancer is the most common diagnosed malignancy in women with over 1 million new cases in the world each year[10]. With an increasing lifetime risk, currently estimated one in eight, it is a leading cause of cancer-related morbidity and mortality. Despite increasing incidence rates, annual mortality rates from breast cancer have decreased over the last decade[11]. Reasons for this decline include, precise diagnosis, increased number of women receiving tailor made treatment, such as extensive use of tamoxifen and the use of chemotherapy and early detection through widespread mammography screening [10;12].

Mammography is currently the most important tool in screening and early detection of breast cancer[10]. In many countries mammography is used as a population based screening in women older than 50 years. However, up to 20% of new breast cancer incidents are not detected by this method [13-15]. Furthermore, only one out of three lesions positively detected using mammography turns out being malignant. Mammography is also used as a screening tool in young women with a high familiar risk or with a genetic predisposition. In this group the detection rate is only 40% mainly because of the dense breast tissue[16;17]. Adding MRI to mammography screening for these at risk patients has good potential to detect mammographically occult cancers but this expensive imaging technique does not reliably distinguish benign from malignant findings and has a high false positive rate [18-20]. Consequently, MRI and also mammography screening can lead to overdiagnosis and overtreatment [18;21], indicating a need for novel molecular markers that might improve specificity and sensitivity for early detection of breast cancers, suitable for population screening or more intensified screening programs for young women with an increased risk.

Colorectal cancer

Colorectal cancer (CRC) is among the most common malignancies and remains a leading cause of cancer-related morbidity and mortality. There are approximately one million new cases of CRC per year worldwide[22]. Although the incidence of CRC fortunately decreases in the United States [9;23], in most countries the incidence

rates increase, particularly due to increase in total population and aging of the current population. In Asia, Eastern Europe, Israel, and Puerto Rico the increase is most dominant. Colorectal cancer arises from a multistep sequence of genetic alterations that results in the transformation of normal mucosa to a precursor adenoma and ultimately to carcinoma. Early detection appears to be the most influential factor to reduce disease-related mortality and treatment related morbidity [23;24]. Unfortunately, at this moment only about 37% of CRC remain localized at the time of diagnosis [25]. Survival in CRC is directly related to the stage of the disease at the time of diagnosis. When cancer is found early at localized stage (stage I), 5-year survival is approximately 95% [9;26] whereas the overall 5-year survival rate of CRC with distance metastasis to distance is less than 5 percent. Early detection by population wide screening programs thus becomes more important.

Access to screening programs varies throughout the world, from population programmatic screening in developed countries to regional level screening programs or the opportunity of having a screening test when entering a health care system. Screening programs in most countries include average risk individuals aged between 50 and 75 years [22] and vary widely in screening incidence as well as method of choice.

Currently available tests used for screening include; guaiac-based fecal occult blood test (gFOBT), immunochemical fecal occult blood test (iFOBT), colonoscopy and flexible sigmoidoscopy (FS). Other less common used tests are stool DNA testing (sDNA), computed tomography colonography (CTC) and double-contrast barium enema (DCBE).

Due to its low costs and easy access, the most frequently used screening method is gFOBT. It detects the peroxidase reaction of hemoglobin. Disadvantages are the false-positive rates which make dietetic provisions necessary and low sensitivity rates from 20-40 % [27].

With iFOBT no dietetic restrictions are necessary because it only reacts to human hemoglobin. A wide range of qualitative and quantitative tests is presently available, with varying levels of sensitivity and specificity. With only one test sensitivity rates are approximately 65%, when repeated every two years sensitivity increases till 80-90 % [28;29].

FS is an endoscopic examination with maximum reach to the splenic flexure. Its sensitivity is about 60-70% for adenomas and CRC [30;31]. Unlike FS, colonoscopy also detects lesions in the proximal colon. Its biggest advantage is the possibility of

removing pathological lesions within a single examination. The sensitivity in detecting both adenomas and carcinomas seems to be high but data from prospective, randomized trials are limited. Also it is an invasive method with a higher risk of serious adverse events than for FS respectively 3-5% compared to 0% to 0.03% [30;32;33]. To implement colonoscopy into national screening programs a huge increase in care capacities is necessary.

sDNA examines the stool for the presence of abnormal DNA. The test sensitivity for CRC ranges from 52% to 91% [27;34-36]. Another disadvantage is its high price.

CTC shows lesions in the colorectum by reconstructing two- and three-dimensional images. To date, no studies have been published assessing reduction in CRC incidence or mortality. DCBE shows the entire colorectum, although with significantly lower sensitivity and specificity than colonoscopy or CTC. The percentage of undetected carcinomas is up to 22% [33].

No available CRC screening test is yet perfect, either for cancer detection or adenoma detection. Each test has associated limitations and risks. There is a great need for alternative, non-invasive methods with high sensitivity and specificity rates, easily available and cost effective. Use of MS based proteomic serum biomarkers could form a specific, more sensitive and less invasive alternative.

WORKFLOW IN PROTEOMIC PROFILING

Blood sample preparation

Human blood is a suitable source of proteins and can be obtained in a relatively easy fashion. Both plasma and serum samples, obtained from whole blood, have been used in biomarker discovery studies. Serum resembles plasma in composition but lacks the coagulation factors. Although serum is preferred for many tests because the anticoagulants in plasma can sometimes interfere with the method, plasma seems to be more stable than serum and more suitable for analysis of the low-molecular-weight proteome. It has been reported by various authors that protein profiles obtained from plasma and serum differ and unfortunately at this time it would appear that insufficient information is available to decide whether serum or plasma should be preferred in MS-based proteomics studies aiming for biomarker discovery. While most studies have been carried out using serum, further research on this topic is required. A temporary solution would be to use both, however this would complicate data analysis and require longer processing times.

Standardization

As is the case for all diagnostic tools in a clinical setting, MS-based proteomic profiles should be precise and accurate, and the methodology needs to be robust and reproducible. Some critics have argued that discriminating peaks are influenced by various factors. Possible confounding factors can be categorized into three sources of variation and bias: biological variation, pre-analytical variation and analytical reproducibility. Examples of biological variation are a.o.; race, age, diet, smoking but also stress, drugs and general physical conditions [37-39]. To date no studies have been reported taking into account these latter aspects. Some groups have reported data on the effects of different sample preparation procedures. In all studies the importance of sample handling was indicated; i.e. the time between blood sampling and serum centrifugation. A delay of 2 to 4 hours seems to be acceptable. De Noo et al. analyzed pre-analytical variables and reproducibility on a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) approach.

It is now generally recognized that standardized sample collection is required for clinical studies[40;41]. In addition, it is recommended that the number of freeze/thaw cycles is kept as low as possible. Finally, it was found that circadian rhythm was not an influencing factor, in other words samples can be collected all over the day. Both the acceptable delay time before serum centrifugation and the ability to collect samples all over the day increases future clinical applicability [38].

The Human Proteome Organisation (HUPO) is an international scientific organization representing and promoting proteomics through international cooperation and collaborations by fostering the development of new technologies, techniques and training. (www.hupo.org). For this review interesting HUPO initiatives are the HUPO Plasma Proteome Project (HPPP) and Human Proteome Project (HPP). A goal of the first initiative is to organize more standardized procedures regarding the collection and measuring of the samples and data processing. An overview of the HPPP results from the pilot phase with 35 collaborating laboratories and multiple analytical groups, generating a core dataset of 3020 proteins and a publicly-available database[42]. The mission of the HPP is to characterize all 21 000 genes of the known genome, thus generate the map of the protein based molecular architecture of the human body and become a resource to help elucidate biological and molecular function and advance diagnosis and treatment of diseases[43].

Clean-up procedure

Human body fluids such as serum are complex mixtures of salts, lipids, peptides and proteins. To carry out a repeatable and robust mass spectrometric analysis of proteins

in body fluids a clean-up or extraction procedure is required [44]. In general protein separation techniques are based on different physical properties of a protein, such as size, iso-electric point, solubility and affinity. Furthermore, the use of a specific agent to capture proteins enriches the sample and thus improves the lower limits of detection. Obviously, enrichment procedures are of great value to capture so-called low-abundance proteins. Unfortunately, low-abundance proteins are often not circulating freely but are a-specifically bound to high-abundance proteins, such as albumin. As a result proteins present at low concentration can be lost in depletion methods [9].

Functionalized magnetic beads

The last decade multiple studies have been carried out using magnetic beads as a method for off-line serum peptide/protein capture [38;45-47]. Magnetic beads are uniform beads specifically designed for quick manual or automated fractionation of proteins or peptides from complex biological samples. This solid-phase extraction (SPE) procedure is quick and simple, sample preparation occurs without the need for laborious pipetting and centrifugation. As mentioned earlier, protein separation techniques are based on different physical properties of a protein. Materials known from these different chromatographic platforms are coupled to the surface of spherical magnetic beads. Magnetic beads described most applied in studies are WCX (weak cation exchange), RPC18 beads (reversed phase) and C8. WCX beads separate proteins based on charge, whereas RPC18 beads separate proteins and peptides via strong hydrophobic interaction[48].

Automation

The manually fractionation and processing steps are tedious and time consuming to perform. Automation ensures reproducibility and facilitates high-throughput performance necessary for large scale studies. In the last few years our study group developed a reliable automated technique that is specially designed for high-throughput sample handling, i.e. processing hundreds of serum samples per day. The activation, wash and desorption steps of WCX and RPC18 beads are based on the manufacturers protocol, with adjustments to allow for optimal implementation on a 96-channel Hamilton STARplus® pipetting robot. With this liquid handling robot, the whole serum peptide/protein capture procedure is automated. Spotting onto a MALDI target plate is carried out in quadruplicate using the same robot.

MALDI-TOF mass spectrometry

Mass spectrometry (MS) is the method of choice for the analysis of proteins in serum [8]. A mass spectrometer separates peptides or proteins according to their mass-to-charge ratio. A mass spectrometer consists of an ion source, a mass analyzer and

a detector. There are several types of mass spectrometers, the one mostly used in profiling strategies is MALDI-TOF. To carry out a MALDI-TOF mass analysis a small amount of specimen containing peptides and proteins is dried on a target plate after mixing with a light-absorbing matrix. MALDI-TOF MS is a rapid biomarker discovery tool that allows high-throughput screening through automated sample processing and profiling.

SELDI-TOF

As an alternative, surface enhanced laser desorption ionization-time-of-flight (SELDI-TOF) can be used[49]. In SELDI-TOF a surface modified with a chemical functionality on a chip is used. A sample clean-up is then carried out on this chip similar to workup with functionalized magnetic beads. Some proteins in the sample bind to the chip surface, while the others are removed by washing. After washing the spotted sample, the matrix is applied to the surface and allowed to crystallize with the sample peptides. Common surfaces include CM10 (weak-positive ion exchange), H50 (hydrophobic surface, similar to C6C12 reverse phase chromatography), IMAC30 (metal-binding surface), and Q10 (strong anion exchanger). Surfaces can also be functionalized with antibodies, other proteins or DNA. Samples spotted on a SELDI surface are mass analyzed using TOF-MS as used in MALDI-TOF.

Combined with magnetic bead fractionation, MALDI-TOF has higher throughput than SELDI-TOF and is more sensitive, as spherical particles have larger surface areas and higher binding capacity than chips. Thus, in SELDI-TOF more serum is necessary for analysis.

Data-analysis

Next to standardized sample collection and preparation protocols the data analysis is of major importance. In 2008 our group organized a competition on clinical mass spectrometry based proteomic diagnosis. Eleven international statistical groups participated and constructed a diagnostic classification rule for allocation of future patients on a blinded calibration set. This classification rule was than tested on a blinded validation set. A variety of statistical methods was used to create a classification rule. Mertens and co-workers described a method in which classification error rates were estimated and validated based on a classical Fisher linear discriminant analysis through complete double cross-validation [50]. Each sample was assigned to the group for which the probability was highest. Other groups for example used the random forest classification method, or a three-step approach with ranking of the mass/charge values using random forests, then grouping into new variables and finding a discriminating rule by penalized logistic regression. For further details and additional

statistical methods see http://www.bepress.com/sagmb/vol7/iss2. This competition showed that a discriminating profile could be created independently of the chosen statistics with consistent results of 80% accuracy [51].

POTENTIAL MASS SPECTROMETRY DERIVED BIOMARKERS

Early detection of breast cancer

Several studies have used mass spectrometry (MS) on serum samples in an attempt to find biomarkers for early diagnosis of breast cancer using the SELDI-TOF [52-58] or MALDI-TOF approach [45]. All studies were case-controlled, except for the study by Mathelin et al. The various studies included sample sizes from 40 to 109 cancer patients with control groups of equal size. Results were encouraging with high sensitivity and specificity rates, varying from 80 to 100%. Several discriminatory peaks were described, such as a peak at 8.9 kDA [52;53;56;59], 4.3 kDa [56-58] and one at 8.1 kDa [53;56].

However, the reproducibility of these results has been questioned. Li et al. identified three peaks associated with breast cancer, termed BC1 (4.3 kDa), BC2 (8.1 kDa) and BC3 (8.9 kDA) [56]. The combination of these three biomarkers allowed differentiation of cancer patients and non-cancer controls with a sensitivity of 93% and specificity of 91%. Mathelin et al. tried to validate these three biomarkers in a set of 49 breast cancer patients and 13 patients with benign breast tumors and 27 controls [58]. Although, both of these studies used SELDI-TOF and nickel-loaded proteinchip arrays, Mathelin et al. could not identify the BC2 peak in their patient series. A combination of BC1 and BC3 could only identify 45% of all breast cancer patients successfully. This is a somewhat disappointing result that might indicate that results obtained in one laboratory are difficult to reproduce in another laboratory or setting. Although limited information concerning handling protocols was provided in the reports of these two studies, differences in methods might have been responsible for this lack of reproducibility [60]. Remarkably, another study found that the peak at 8,9 kDa was decreased, whereas in other studies this peak was increased [61]. Even a follow-up study by the same group could not reproduce the BC1 peak [62].

All described differences can be due to modification of peptides or proteins between the moment of sample collection and freezing, which has been described [63]. Some of these studies used different methods than others, with regard to time between collection and freezing, time of centrifugation and storage freezing temperature which

may well lead to variability in outcome. Results by Fan et al. were more optimistic. This study tested a classification model after initial identification in a different patient group. On a blinded patient population this model had high sensitivity and specificity (96,45% and 94,87% respectively) [53], indicating a good reproducibility if MS is performed under the exact same conditions. (Table 2.)

Early Detection of CRC

Mass spectrometry has been applied for the development of tests for early diagnosis of CRC in several studies [64-70]. All of these were case-control studies and so far no prospective or randomized studies have been reported. Published studies reported promising results and underline the potential of mass spectrometry for early diagnostics. Patients diagnosed at several stages of colorectal cancer were included these studies (Dukes stage I to IV). Although for all mentioned studies serum samples were used, the applied methods differed. Only de Noo et al used MALDI-TOF MS in combination with C8 magnetic beads, while all others used the SELDI-TOF system with varying detection chips. For instance, Engwegen et al found the best results by using CM10 chips, while Liu et al. compared obtained serum profiles with several chips and found the best results with the IMAC30 chip with the SELDI-TOF system. More research has to be done to optimize pre-analytical and detection variables. However, Ward et al and Liu et al found reproducible results when identical methods and materials were used. Many studieshowever present variations in methods for storage and handling of the serum samples, the time period between sample collection and freezing and samples were stored at different temperatures.

The aforementioned studies used discriminant analysis to discriminate between cancer patients and healthy controls. Interestingly, several peaks were repeatedly found in multiple studies signifying their potential as a biomarker. For instance, a peak at m/z ratio 8940 Da (identified as complement protein C3a-desArg) was found by Ward et al, Habermann et al, Zhao et al and Yu et al. Another peak at 5911 Da was used as a discriminating peak both by Yu et al and by Chen et al.. Most studies tried several combinations of significant peaks and used those to identify cancer patients. All studies were capable of achieving sensitivity and specificity values of around 90% or higher. However, we have to be cautious since these results might be overoptimistic.

Since some of the algorithms were tested on the same group of patients which was used to create the algorithm, results might be biased. Also, relatively small groups were used in these studies (40 to 60 colorectal cases with control groups consisting of a similar size of healthy controls). Validation of these results on a larger and independent patient group is therefore necessary. Some of the published studies used

a (small) independent group for validation of the sensitivity and specificity [65;68;71]. Engwegen et al. validated their classification tree on independent patient samples, from which a test sensitivity and specificity of 66.7% and 89.5% were found. Liu et al. found a sensitivity of 95% and specificity of 94.87% when testing their biomarkers on a set of 60 cancer patients and 39 healthy subjects. (Table 1.)

IDENTIFICATION OF BIOMARKERS

The first studies investigating the possibility of early diagnosis of breast and colorectal cancer with mass spectrometry did not include the identifications of the discriminating peaks. Ideally, these would all be proteins produced by tumor cells only and secreted into the blood in sufficient quantities to be detected. The identification of discriminatory proteins has become an important element in recent studies and will be discussed below.

Identifying proteins is by no means simple and requires additional analytical tools. In the early days, MALDI-TOF mass fingerprinting was used for MS-based protein identification. To this end, a protein is enzymatically converted into peptides, typically with trypsin. Since the tryptic digestion is highly site-specific the identification of at least two peptides allows identification of the original protein. This method, however, only works for purified proteins. Nowadays, the method of choice for protein identification is tandem mass spectrometry (MS/MS or MS2). The tryptic peptides are first separated using high-performance liquid chromatography (HPLC) before performing MS/MS identification. The HPLC is interfaced with a tandem mass spectrometer through an electrospray ionization (ESI) source. So-called LC-MS/MS methods are highly suited and optimized for peptide sequencing. Sequencing experiments (i.e. MS/MS) are carried out on ions that are selected in a prescan (i.e. MS). Peptides are collided with inert gas which causes these peptides to fragment, resulting in product ions that can be interpreted with respect to the primary amino acid sequence. The resulting spectra are used to identify the peptides in question. This can be done in various ways; by de novo sequencing or by spectral matching using databases. With de novo sequencing, the amino acid sequence of a peptide is reflected in the fragment ion mass spectrum. The mass difference between two neighboring peaks is equal to the mass of one amino acid. However, when not all peptide bonds are broken or when not all expected fragment ions appear in the mass spectrum, interpretation may be ambiguous. Therefore, spectral matching is more frequently used. This method identifies peptides by comparing an MS/MS-spectrum with theoretically expected peptide spectra that are stored in a database. After comparison the best matching peptide(s)

Table 1. Early Detection of CRC

Study	MS Method	Study Size N=	Sensitivity	Specificity	External validation?
Yu et al. World J Gastroenteroloy 2004	SELDI-TOF	55 CRC 35 CRA 92 HC	89%	83 - 92%	No
Liu et al. Cancer Investigation 2006	SELDI-TOF	74 CRC 48 HC	95%	94.87%	Yes N= 60 CRC, 39 HC
De Noo et al. European Journal of Cancer 2006	MALDI-TOF	66 CRC 50 HC	95.2%	90.0%	No
Ward et al. British Journal of Cancer 2006	SELDI-TOF	62 CRC 31 HC	95%	91%	No
Chen et al. Clinical Cancer Research 2004	SELDI-TOF	55 CRC 92 HC	91%	93%	No
Zhao et al. Chinese Journal of Clinical Medicine 2004	SELDI-TOF	73 CRC 16 CRA 31 HC	96%	98%	Yes N= 73 CRC, 16 CRA, 31 HC
Engwegen et al. World Journal of Gastroenterology 2006	SELDI-TOF	77 CRC 80 HC	66.7 - 89.5%	73.3 - 88.9%	Yes

CRC = colorectal adenoma, CRA = colorectal adenoma, HC = healthy controls

However, the reason these techniques are being developed is for screening in patient populations where the a priori chance of having colon cancer is much smaller than in the patient series in these studies. With a lower a priori chance, the positive predictive value will likely be lower. First trials on large representative patient populations or patients with an increased risk of colorectal cancer are therefore essential.

Table 2. Early detection of Breast Cancer

Study	MS Method	Study Size (N)	Sensitivity	Specificity	External Validation?
Hu et al. The Breast 2005	SELDI-TOF	49 BC 51 BBD 33 HC	83.33%	88.89%	Yes N=18 BC, 9HC
Fan et al. Cancer Research Clinical Oncology 2010	SELDI-TOF	80 BC 40 HC	96.45%	94.87%	Yes N= 44 BC, 98 BBD, 20 HC
Belluco et al. Annals of Surgical Oncology 2006	SELDI-TOF	109 BC 109 HC	95.6%	86.5%	Yes N= 46 BC, 46 HC
Callesen et al. Journal of Proteome Research 2008	SELDI-TOF	48 BC 28 HC	85%	85%	No
Li et al. Clinical Chemistry 2002	SELDI-TOF	103 BC 25 BBD 41 HC	93%	91%	No
Vlahou et al. Clinical Breast Cancer 2003	SELDI-TOF	45 BC 42 BBD 47 HC	80%	79%	No
De Noo et al. Onkologie 2006	MALDI-TOF	78 BC 29 HC	100%		No

BC = Breast cancer, BBD = Benign Breast Disease, HC = healthy controls

Like in colorectal cancer, the size of the investigated groups was relatively small. Some studies found MS to be able to differentiate benign from malignant abnormalities [52], but most studies used healthy people as controls which obviously is not representative for the general patient population.

are given together with a score indicating the closeness of the match. This database (SpectraST for example) consists of a collection of theoretical spectra that are derived from all possible proteins that can originate from the genome. These databases take certain splice-variants into account, however the existence of alternative splice variants or mutations related to for instance cancer hampers the identification of peptides. A related problem with this approach is the redundancy of proteins that do not actually occur but that increases the chance of accidental matches. Another possibility is matching the spectrum of product ions to spectra that were obtained from standard (synthetic) or previously identified peptides. This approach has the advantage that the database does not include any proteins that are not naturally present and thus decreases the number of false positives. Obviously, the disadvantage is that it can not be used to identify proteins that are not included in the database. Note the difference between peptide- and protein identifications. The peptides are identified directly from the MS/MS-spectra, with a certain confidence, whereas a protein identification is derived from a combination of multiple peptide ID's. Several parameters exist to express the reliability of the peptide and resulting protein matches. The mathematics and statistics that are used for this purpose fall beyond the scope of this review but are reviewed elsewhere [72]. The reliability of protein identifications can be increased by using known properties of the yet unidentified protein. For instance, if the protein is also analyzed on SDS-PAGE, its mass can be identified and proteins that have a different mass can be left out of the database analysis. Some studies have used western blotting to identify proteins on SDS-PAGE after identification which is an effective method to confirm protein identity, if reliable antibodies are available.

Biomarker identification in breast cancer

Only limited studies have identified biomarkers in MS studies for breast cancer. Li et al. tried to identify their previously identified BC1-3, but could only identify BC2 and BC3 as fragments of C3a, desArg [62]. This protein was also identified in colorectal and MS studies in other forms of cancer, The BC1 is suspected to be interalpha-trypsin inhibitor heavy chain H4. Fan et al. found apolipoprotein C-I to be down-regulated in breast cancer patients. The two other discriminatory peaks were identified as C-terminal-truncated form of C3a and complement component C3a [53]. Like in colorectal studies, the so-far identified proteins might seem to be lacking in specificity as these are not tumor-produced proteins. However, Villanueva et al. described not only cancer-specific, but cancer type-specific biomarkers [63]. The strength of this study was that it was the first to not only take the identity of the potential biomarkers but also realize the importance of the biomarkers' mass. This study found 11 unique biomarkers for breast cancer compared to prostate cancer and bladder cancer patients. These were all protein fragments cleaved from proteins normally present in the serum (fibrinogen

α, C4a, C3f, ITIH4, ApoA-IV and transthyretin). Further research into these 11 biomarkers might find a set of unique biomarkers for breast cancer. It therefore seems that the biomarkers that are discovered with MS are not tumor-specific proteins, but tumor-specific protein fragments. This may be likely due to tumor-specific secretion of proteases which cleave high-abundant serum proteins. (Table 4.)

Table 3. Biomarker identification in colorectal cancer

Author	Identified biomarkers (m/z ratio)
Engwegen et al. World Journal of Gastroenterology 2006	- N-terminal albumin fragment ($3,1x10^3$) - Apolipoprotein C-I ($3,3x10^3$ / $6,6x10^3$) - Apolipoprotein A-I ($28x10^3$)
Ward et al et al. British Journal of Cancer 2006	- Alpha1-antitrypsin ($50,7x10^3$) - Apolipoprotein C-I ($6,4x10^3$/ $6,6x10^3$) - Transferrin ($79,1x10^3$), - C3 fragment ($8,94x10^3$)
Albrehtsen et al. BMC Cancer 2005	- HNP 1 ($3,37x10^3$) - HNP 2 ($3,44x10^{3)}$) - HNP 3 ($3,49x10^3$)

Table 4. Biomarker identification in breast cancer

Author	Identified biomarkers (m/z ratio)
Li et al. Clinical Chemistry 2005	- C3 fragment ($8,1x10^3$ / $8,9x10^3$)
Fan et al. Journal of Cancer Research and Clinical Oncology 2010	- Apolipoprotein C-I ($6,6x10^3$) - C3 fragment ($8,1x10^3$ / $8,9x10^3$)
Villanueva et al. Journal of Clinical Investigation 2006	- FPA, fibrinogen alpha, C3f, C4a, ITIH4, ApoA-IV, Bradykinin, Factor XIII, Transthyretin

Biomarker identification in colorectal cancer

One of the most frequently found potential biomarkers, C3a-desArg is not a tumor secreted protein, but a component of the complement system. Elevation of this protein is therefore more likely to be a reflection of the body's inflammatory response activated by cancer. Interestingly, using serum ELISA testing of C3a-desArg levels, Habermann et al. were able to identify cancer patients with a sensitivity of 96,8% and specificity 96,2%. However the control group in this study consisted of healthy medical personnel. This group was not age matched and might therefore have a relatively lower chance of additional diseases than the screening population aged 50-75 yrs. which might lead to nonspecific elevation of C3a-desArg levels. For instance, Li et al. also reported an elevation of C3a-desArg in patients with breast cancer [62]. This implies that the elevation of these proteins is in fact non-specific and has little value in early identification of colorectal (or breast cancer) [73]. Another identified protein by Ward et al. was a peak at m/z ratio 5070 Da, which was identified as α1-antitrypsin

and is involved in the immune response. It has also been implicated in other forms of cancer, so this is unlikely to be a specific indicator of CRC. Albrehtsen et al. found an increase in serum human neutrophil peptides 1,2 and 3 (HNP 1-3) signals compared to controls via Seldi-TOF mass spectrometry. These proteins are involved in regulation of the immune response. HNP 1-3 are found to be upregulated in colorectal cells compared to normal epithelial cells [74]. Testing for CRC by measuring serum levels with an ELISA assay yielded a sensitivity of 69% and specificity of 100% in a group of 26 colon cancer patients and 22 controls. However, the control group consisted of healthy controls only. Because of this, the high specificity is likely to be overoptimistic. Expression of HNP 1-3 has been found in a variety of other tissues, both in inflammatory and neoplastic conditions.

Engwegen et al. found a non-specific increase of discriminating proteins (N-terminal fragment of albumin, apolipoprotein CI, apolipoprotein AI and a yet unidentified protein at 5900 kDa) in other cancer types as well. However, some of these acute phase proteins might be used in combination with other biomarkers that are more specific biomarkers for CRC. For instance, the m/z ratio 5900 Da peak also found by Engwegen et al. (and by Yu et al.was able to discriminate 76% of CRC from other forms of cancer. So far this protein has not been identified. (Table 3.)

DISCUSSION

Numerous studies have described favorable reports on serum protein profiling of breast and colorectal cancer patients. These studies used limited amounts of patients and were generally case-control studies. The fact that the control groups consisted of healthy people has made it impossible to determine whether discriminatory peaks are actually cancer-specific or only "disease-specific". It may be that peaks that are now seen as cancer-specific are in fact due to inflammation or obstruction caused by cancer. Further studies, who not only include healthy persons, but also a control group representative of the general patient population are essential to help to resolve this question. Unfortunately most reports lack reporting detailed information regarding the used control group.

In addition prospective studies are needed to determine the value of MS in the clinical practice. However, before these can take place, more research needs to be done on the reproducibility and optimal handling and processing methods[76]. An ideal set up to apply MS in a routine clinical screening setting in our opinion would be first to validate the profiles in a population screening. Secondly centralized profiling could

be performed in e.g. specialized regional centers. Finally, when discriminating proteins are identified, a simple test (e.g. ELISA) could replace profiling for the identification of cancer patients.

Studies on serum samples have identified several potential biomarkers. Most of the markers that were identified so far were (cleaved) proteins that were present in the serum at relatively high concentrations, i.e. the so-called high abundant proteins (milli-microgram/mL)[77]. In this respect MS faces the challenge of the high dynamic concentration range since tumor-specific proteins are often low abundant (<100 nanogram/mL). In addition, there are indications that the entire spectrum of cleaved proteins by tumor-specific exoproteases can be used to identify patients with cancer. This implies that not only the identity, but also the size of the biomarker is important for accurate diagnosis [78]. Only testing the presence of a certain biomarker is likely to be nonspecific, since this protein might also present in other diseases and other forms of cancer. However, the spectrum of specific fragments of these proteins might be the key to a successful diagnosis instead of conventional single biomarkers. Ironically, the breakdown of these proteins occurs after collection of the serum sample from the patient. This makes it all the more important to have strict guidelines for handling the samples after collection if results are to be reproducible between different centers. All of these results have changed the way of thinking about biomarkers. Finding a single biomarker with MS might be impossible, since all tumors have a different molecular background, but it might be possible to combine several protein fragments to develop highly reliable tests allowing early cancer diagnosis. Although there are doubts on some of these results [79], MS remains a powerful tool in moving forward these discoveries into the clinical practice.

CONCLUSION

In conclusion several methods exist for the early diagnosis of colorectal and breast cancer. Current screening methods have disadvantages like high-cost, invasive nature or insufficient sensitivity or specificity. Because of this, the search for a better diagnostic screening test for both these types of cancer is still ongoing. MS has recently been applied for the identifying serum biomarkers and may lead to a relatively inexpensive (approximately 15 € per sample), minimally-invasive and reliable test for early cancer diagnosis.

Several case-control studies have reported favorable results for diagnosis of breast and colorectal cancer. Comparing the reported sensitivities and specificities of the dif-

ferent research groups with current screening techniques MS would appear to be very promising, with the remark that screening results based on these groups due to the increased a priori chance are likely to be overoptimistic when compared to screening in normal population. In addition these studies used different methods, handling protocols and significantly altered peaks for discriminating between cancer patients and healthy controls. In order to apply MS in a routine clinical setting, collecting, measuring and processing of data will need to be subject to stringent quality control procedures. The current roboting techniques allow high throughput. More comparative studies on influential factors and optimal methods are necessary. Subsequent prospective studies in representative patient populations can then determine whether MS is superior to other screening methods.

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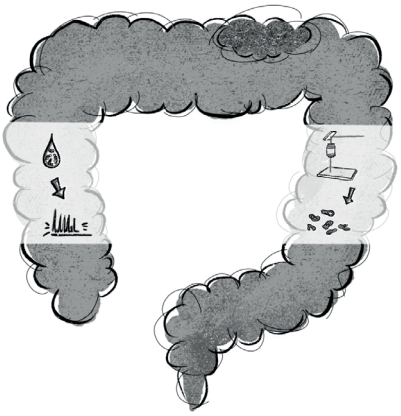

3

Case-controlled identification of colorectal cancer based on proteomic profiles and the potential for screening

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Colorectal Disease 2014 Nov;16(11):907-13.

ABSTRACT

Aim: Colorectal cancer (CRC) screening programmes detect early cancers but unfortunately they have limited sensitivity and specificity. Mass spectrometry-based determination of serum peptide- and protein profiles provide a new approach for improved screening.

Method: Serum samples from 126 CRC pretreatment patients and 277 control individuals were obtained. An additional group of samples from 50 CRC patients and 82 controls was used for validation. Peptide and protein enrichments were carried out using reversed-phase C18 and weak-cation exchange magnetic beads (MBs) in an automated solid-phase extraction and spotting procedure. Profiles were acquired on a matrix-assisted laser desorption/ionization time-of-flight system. Discriminant rules using logistic regression were calibrated for the peptide and protein signatures separately, followed by combining the classifications to obtain double cross-validated predicted class probabilities. Results were validated on an identical patient set.

Results: A discriminative power was found for patients with CRC representative for all histopathological stages compared with controls with an area under the curve of 0.95 in the test set (0.93 for the validation set) and with a high specificity (94-95%).

Conclusion: The study has shown that a serum peptide and protein biomarker signature can be used to distinguish CRC patients from healthy controls with high discriminative power. This relatively simple and cheap test is promising for CRC screening.

INTRODUCTION

The lifetime risk of colorectal cancer (CRC) in the US population is 5–6% without screening, which is similar to the Netherlands (1-3). Early diagnosis reduces disease-related mortality (4). The number of patients diagnosed annually is still increasing, because of aging of the population and a small increase in the incidence at all ages. It is therefore expected that population screening programmes aiming at early detection of CRC will become more relevant. Currently the most promising screening tests used in population screening include the immunology-based faecal occult blood test (iFOBT), DNA markers in stool (sDNA), computed tomography colonography (CTC) and colonoscopy (4;5). The iFOBT uses antibodies to detect the globin portion of human hemoglobin. Multiple brands of tests are available and specificity and sensitivity values reported in literature vary widely from 70% to 94% (6;7). Current advice is annual screening with iFOBT. Screening based on sDNA involves the identification of genetic modifications in the initiation of a sequenced progression from adenoma to carcinoma. The sensitivity and specificity of the various sDNA tests range from 52% to 91% and from 93% to 97% (5). The guideline recommendation is to screen every 3 years. Virtual colonoscopy or CTC is reported to have overall sensitivities of 55-94%, depending on the size of the detected polyps, with high specificity (91-96%) (8). Guidelines advise a screening interval of 5 years. Serum carcinoembryonic antigen (CEA) estimation lacks sensitivity and specificity (9). Although not used for screening, colonoscopy has a specificity and sensitivity of at least 95% for large polyps, but the miss rate for polyps smaller than 5mm is 15–25% and 0–6% for polyps of 10 or more millimetres (10).

Although current screening methods are widely available, there is room for improvement and new developments of simple, cost-effective and noninvasive screening tests (11;12). The use of protein biomarkers for early detection of cancer is promising (13;14). Comparison of serum protein patterns or profiles has allowed the separation of patients with cancer from healthy individuals (15). Alternatively tissue can be used to identify protein biomarkers (16), but results obtained from body liquids and cancer tissue may not be the same. We have developed a one-step, fully automated and standardized solid-phase extraction (SPE) method using functionalized magnetic beads (MBs) to enrich for subsets of peptides and proteins in a high-throughput fashion, in combination with matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF) read-out (16-18). In this study, we use a combination of two different types of paramagnetic beads (MBs) to increase the number of detected features, namely based on weak cation exchange (WCX) and reversed phase (RP) C18-functionalization. Previously, we have shown that the statistical combination of two thus obtained data sets

improves classification of samples in a case-control study of breast cancer patients (19). In the current study, we used MALDI-TOF MS to generate a protein and peptide signature of a serum sample in a case-control set-up aiming for the detection of CRC.

METHOD

Patients

Serum samples within the test set were obtained from 126 outpatients with CRC before treatment and from 277 healthy controls collected between October 2002 and December 2008. Validation specimens were obtained from 50 patients with CRC and 82 healthy controls. These were collected in the same way between January 2009 and May 2010. Histopathological examination of the surgical specimen reported the TNM stage (TNM Classification of Malignant Tumours fifth edition). Informed consent was obtained from all subjects and the study was approved by the Medical Ethical Committee of the Leiden Universal Medical Center.

Sample processing and MALDI-TOF measurement

The method of serum collection, sample and profile processing and data analysis has been described previously (16). The isolation of proteins and peptides from serum was performed using a kit based on magnetic bead purification with WCX- and RP C18 Mbs. using a standardized protocol. High-throughput SPE was followed by MALDI-TOF measurement on an Ultraflex II TOF/TOF spectrometer (Bruker Daltonics). In this way, so-called WCX- and RP C18-profiles were obtained, representing (small) protein and peptide signatures respectively.

Profile processing

For optimal data analysis, all WCX- and RPC18-profiles underwent baseline correction followed by alignment (19). A list of selected peaks (Table 1) was then compiled through the application of a peak selection procedure as previously described by our group (19). In this way, 57 peptides and proteins were selected in the WCX-profiles and 42 peptides in the RP C18-profiles. The summarized spectral measurements for each individual were then used within the discriminant analysis (19).

Statistical analysis

Discriminant rules were calibrated for the WCX and RP C18 data separately using logistic ridge regression (see Appendix S1) (19). A combined classification rule was calibrated using logistic regression on double cross-validation. Predictive performance of the calibrated combination was verified on the validation set. Error rates were

calculated as well as estimates of sensitivity and specificity, assigning each observation to the group for which the predicted class probability was highest, and the receiver operating characteristics (ROC) curve was plotted with the area under the ROC curve (AUC) indicating the ability to distinguish cancer from control samples (Fig. 1).

Table I Summary of all m/z-values used for statistical analysis of the peptide- and protein signatures from the reverse phase (RP C18) and weak cation exchange (WCX) profiles.

m/z value in WCX profile	Window (*m/z* units)*	*m/z*-value in RPC18 profile	No. of peaks†
1866	5	1020.6	3
1898	5	1077.7	3
1947	5	1206.7	3
2024	5	1211.8	3
2084	5	1260.6	3
2106	5	1263.7	3
2661	5	1348.9	3
2756	5	1350.8	3
2770	5	1368.0	3
2862	5	1418.6	3
2884	5	1434.7	3
2953	5	1440.7	3
3159	5	1449.9	3
3192	5	1465.8	3
3241	5	1481.8	3
3269	5	1518.9	3
3328	5	1536.9	3
3445	5	1561.9	3
3501	5	1563.0	3
3525	5	1606.0	3
3539	5	1615.8	3
3884	5	1627.0	3
3956	5	1691.1	3
3972	5	1740.1	3
3994	5	1778.1	3
4054	5	1865.2	3
4090	5	1896.0	3
4210	10	1934.2	3
4480	10	2021.3	3
4648	5	2271.1	3
4963	6	2378.4	3
5065	5	2553.1	3
5087	5	2602.5	3
5160	5	2616.5	3
5248	5	2753.7	3
5336	7.5	2768.4	3
5355	6	2931.5	3
5640	6	2937.8	3
5750	6	3156.8	4
5903	5	3190.6	4
5920	5	3261.7	4
6080	5	3954.2	4
6090	5		
6434	5		
6458	9		
6632	15		
6656	7		
7008	5		
7470	10		
7765	25		
7925	30		
8148	35		
8605	30		
8760	35		
8939	30		
9291	35		
10 270	30		

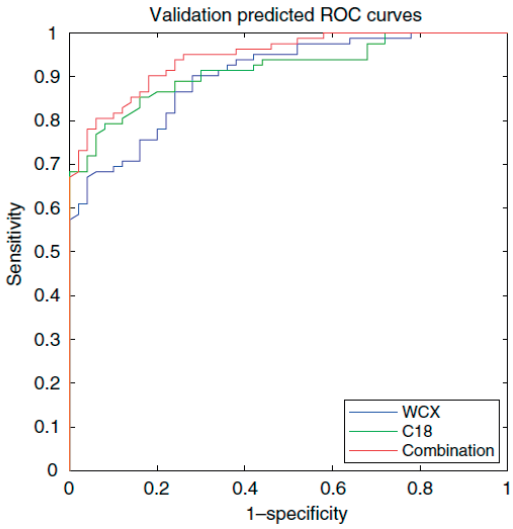

Figure 1 Receive operating characteristic (ROC) curves of the validation set based on weak cation exchange (WCX) and reverse phase (RP) C18 data sets separately and after combination. The area under the ROC curve is a measure of between-group separation (case–control).

RESULTS

Patients

There were 126 outpatients (76 men) with CRC before treatment of median age 65 (25-90) years. The control group included 277 normal individuals (110 men) of median age 56 (24-84) years. The validation set consisted of 50 pre-treatment CRC patients (28 men) of median age 68 (26-91) years and 82 controls (32 men) of median age 45 (21-75) years (Table 2).

Table 2 Patient characteristics.

	Test set		Validation set	
	Patients	Controls	Patients	Controls
n	126	277	50	82
Median age (years)	65	56	68	45
Age range (years)	25–90	24–84	26–91	21–75
Male (%)	76 (60)	110 (40)	28 (56)	32 (39)
Female (%)	50 (40)	167 (60)	22 (44)	50 (61)
Stage (Dukes)				
A (%)	22 (18)	NA	7 (14)	NA
B (%)	52 (41)	NA	15 (30)	NA
C (%)	28 (22)	NA	18 (36)	NA
D (%)	24 (19)	NA	10 (20)	NA

NA, not applicable.

Peptide- and protein signatures

In total 535 serum samples (test- and validation set) were processed with two types of magnetic beads. MALDI-TOF profiles were obtained in quadruplicate, yielding 2140 WCX- and 2140 RPC18-profiles. Profiles were baseline-corrected, aligned and of the mean of the four quadruplicates was calculated. Low-quality profiles (approximately 1%) resulting from a failure in sample processing or failed MALDI-spotting were excluded from analysis. The strategy for data analysis and statistical evaluation is shown in Fig. 2. First, all peptide- and protein profiles, obtained from RP C18 and WCX workup procedures were aligned to the *m/z*-axis. Then 42 and 57 peaks (summarized in Table 1) were selected from the RP C18- as well as WCX profiles indicating patient samples (in green) and controls (in blue). In this way, two data sets were obtained that were used for statistical analysis. In the combination plot of the patient samples the correctly classified cases are in green, whereas the remaining wrongly classified cases are in red. From this plot it can be seen that 18 of the 50 cases were incorrectly classified. The combined classification results of the control samples show that all were correctly classified (in blue) (Fig. 2, bottom right). From this plot it can be seen that only 4 of the 82 control samples were incorrecty classified as "cases" (in red). The clinical background of incorrectly classified patient and control samples was further evaluated and the results are summarized in Table 3.

Figure 2 Overview of study design and classification results. On the left-hand side typical examples of peptide (reverse phase, RP C18) and protein (weak cation exchange, WCX) profiles are shown, cases are in green and controls in blue. From all RP C18 and WCX profiles 42 and 57 peaks, respectively, were selected for statistical analysis. The results for the validation set are plotted on the right-hand side and further explained in the Results.

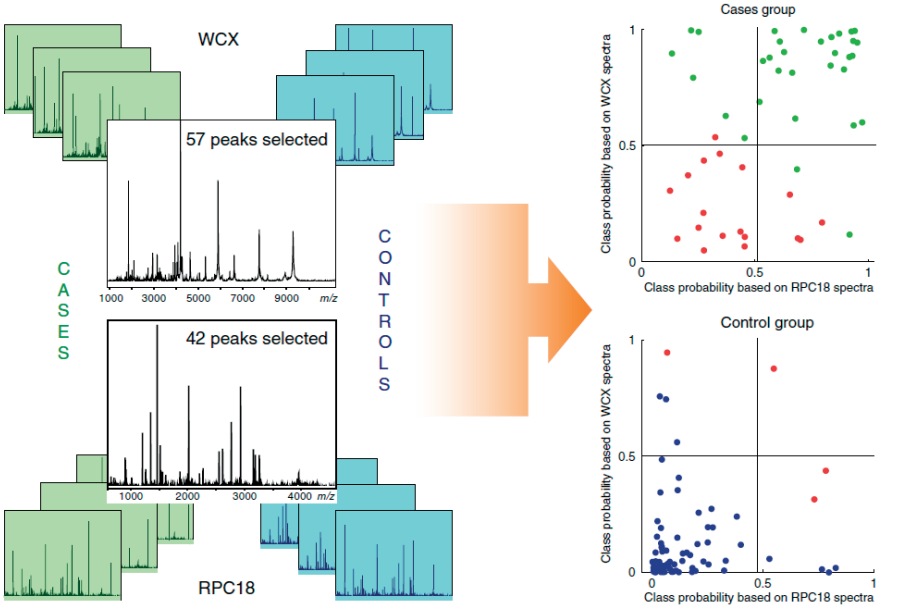

Table 3 Characteristics of misclassified cases for different cut-off values. 'Misclassified cases' in the case group are patients with colorectal cancer (CRC) with the specific cut-off value who were misclassified as controls, whereas 'Misclassified cases' in the control group represent controls with the same cut-off value were misclassified as CRC patients.

	Cut-off value							
	0.5		0.3		0.2		0.1	
	Case	Control	Case	Control	Case	Control	Case	Control
(a) Characteristics of misclassified cases and controls in the test set (total number of cases and controls 126 and 277)								
Total misclassified (%)	23 (18)	16 (5.8)	13 (10)	36 (13)	10 (7.9)	51 (18)	4 (3.2)	71 (26)
Sensitivity	0.817		0.896		0.921		0.968	
Specificity	0.942		0.870		0.816		0.754	
Gender								
Male (%)	19 (83)	9 (56)	12 (92)	17 (47)	9 (90)	21 (41)	4 (100)	27 (38)
Female (%)	4 (17)	7 (44)	1 (8)	19 (53)	1 (10)	30 (59)	0 (0)	44 (62)
Age (years)								
Median	60	63	57	60	57	57	56	57
Minimum	50	38	52	35	52	35	55	35
Maximum	82	80	82	82	82	84	74	84
Dukes								
A	5	NA	3	NA	2	NA	1	NA
B	8	NA	4	NA	4	NA	1	NA
C	7	NA	5	NA	3	NA	2	NA
D	3	NA	1	NA	1	NA	0	NA
(b) Characteristics of misclassified cases and controls in the validation set (total number of cases and controls 50 and 82)								
Total misclassified (%)	18 (36)	4 (4.9)	12 (24)	7 (8.5)	9 (18)	11 (13)	2 (4.0)	21 (26)
Sensitivity	0.640		0.760		0.820		0.960	
Specificity	0.951		0.914		0.866		0.780	
Gender								
Male (%)	9 (50)	2 (50)	5 (42)	2 (29)	4 (44)	2 (18)	0 (0)	6 (29)
Female (%)	9 (50)	2 (50)	7 (58)	5 (71)	5 (56)	9 (82)	2 (100)	15 (71)
Age (years)								
Median	68	34	71	33	67	35	62	41
Minimum	39	26	39	23	39	23	57	23
Maximum	86	51	86	52	82	52	67	69
Dukes								
A	7	NA	6	NA	5	NA	1	NA
B	2	NA	1	NA	1	NA	0	NA
C	6	NA	4	NA	2	NA	0	NA
D	3	NA	1	NA	1	NA	1	NA

NA, not applicable.

DISCUSSION

In this study we have evaluated mass spectrometry-based peptide and protein signatures for improved early cancer detection, motivated by that fact that the success rate of currently available tests for early diagnosis of CRC is rather low (11). These signatures, or profiles, were used successfully to distinguish CRC patients from healthy controls with a high discriminative power. It was found that the applied technology is a potential candidate for screening and early detection of CRC.

Despite the high discriminative power larger studies are essential (and on-going) to investigate the "tumour-specificity" of the obtained discriminating signatures. Survival

is relatively good when CRC is diagnosed in an early stage (3). Early detection will identify cancer when it is still localized and curable, not only preventing mortality, but also reducing morbidity and costs. Detection of symptomless CRC or precursor lesions through population screening allows for more effective treatment, which would likely improve long-term survival (3;4).

Full automation of the preparation and analysis process ensures standardization and robustness together with high discriminative power, supporting the potential of this test for screening programs. Encouraging results of well above 90% were obtained with regard to specificity- and sensitivity values. Cut-off levels can be chosen depending on the defined strategy for patient management and availability of colonoscopy facilities. Implementing a test for population screening requires consideration of factors such as compliance and costs. Enhanced sensitivity is an essential goal in the development of a screening test; however the fine-tuning of the ideal cut off value also depends on the organisation of the healthcare environment. False positive results are associated with patient anxiety and unnecessary colonoscopy (20). Zorzi and co-workers (21) evaluated five (large) population screening programs using iFOBT, that reported a total of 267,769 screened individuals of which 13,388 (5.0%) had a positive iFOBT test. From this group 90.3% (12,089 persons) were followed-up with colonoscopy, of which 748 individuals (6.2%) had a screen detected cancer. Thus, more than 90% of the persons with a positive iFOBT resulted in a negative colonoscopy (21).

Colonoscopy is an invasive procedure with a complication rate of 0.8-2% (22;23), which often requires sedation, which includes monitoring, extra nursing support and risk. Furthermore colonoscopy is time consuming and not really suited for screening. Both colonoscopy and CTC require bowel preparation and have a low participation rate of respectively 22% and 34% (1). The serum proteomics test is based on the analysis of one tube of peripheral blood, which is more convenient for the patient.

In conclusion, serum protomics analysis is easy to apply, cheap and patient-friendly with good sensitivity and specificity. The next step is to compare the test performance to current screening methods such as iFOBT. To this end, population screening will be evaluated, comparing serum proteomics analysis with iFOBT using colonoscopy as the gold standard. This may ultimately result in new guidelines for CRC screening in the Netherlands.

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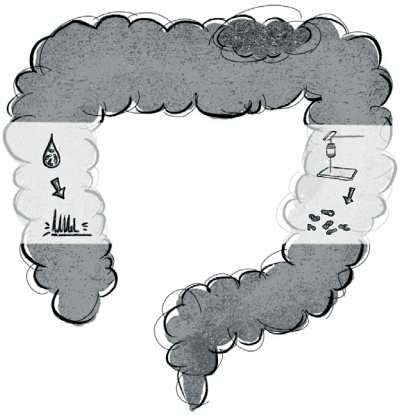
APPENDIX S1. DISCRIMINATION BETWEEN PATIENTS AND CONTROLS

Both the WCX and RPC18 data were calibrated separately with a discriminant rule using logistic ridge regression based on the training- or test set. Joint calibration of the classifiers and unbiased estimation of the class probabilities on the training set was achieved using double cross-validation, as described previously (26). The two sets of double cross-validated class probabilities which were thus obtained on the training data were then used as inputs for the estimation of an ordinary logistic regression model, which combines the predictions from the WCX and RPC18 training data. To evaluate this combination classifier, first the logistic ridge regression models were refit on the WCX and RPC18 data separately using the optimum penalty term identified in the previous double cross-validatory analysis. Then, for each validation sample these two logistic regression models were applied to obtain class predictions on the WCX and RPC18 sets separately. Finally, these two predictions were combined using the above described ordinary logistic regression combination model, which gives a single output class probability for each individual in the validation data. The double cross-validated results yielded a total recognition rate with an AUC of 0.95. For the validation set the AUC was 0.93 (see Figure 2). Different cut-off values were evaluated to match the most optimal test performance in a given population with respect to the colonoscopy capacities/facilities, as is summarized in Table 3. As an example, at a cut-off value of 0.5 the sensitivity and specificity numbers in the test set are 82% and 94% (validation: 64% and 95%), whereas at a cut-off of 0.2 the sensitivity and specificity are 92% and 82% (validation: 82% and 87%). A low cut-off value results in optimal sensitivity at the cost of specificity. Whereas when a higher specificity is preferred a higher cut-off value can be chosen. In Tables 3A and 3B an overview is given of the patient characteristics, which were misclassified in this study design at an associated chosen cut-off value.



4

The proportion of tumor-stroma as a strong prognosticator for stage II and III colon cancer patients; validation in the VICTOR trial.

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Annals of Oncology 2013 Jan;24(1):179-85.

SUMMARY

Background: The intra-tumor stroma percentage in colon cancer (CC) patients has previously been reported by our group as a strong independent prognostic parameter. Patients with a high stroma percentage within the primary tumor have a poor prognosis.

Patients and Methods: Tissue samples from the most invasive part of the primary tumor of 710 patients (52% Stage II, 48% Stage III) participating in the VICTOR trial were analyzed for their tumor-stroma percentage. Stroma-high (>50%) and stroma-low (≤50%) groups were evaluated with respect to survival times.

Results: Overall and disease free survival times (OS and DFS) were significantly lower in the stroma-high group (OS $p<0.0001$, Hazard ratio (HR)=1.96; DFS $p<0.0001$, HR=2.15). The five year OS was 69.0% versus 83.4% and DFS 58.6% versus 77.3% for stroma-high versus stroma-low patients.

Conclusion: This study confirms the intra-tumor stroma ratio as a prognostic factor. This parameter could be a valuable and low cost addition to the TNM-status and next to current high-risk parameters such as Microsatellite instability (MSI) status used in routine pathology reporting. When adding the stroma-parameter to the ASCO criteria the rate of "undertreated" patients dropped from 5.9% to 4.3%, the "overtreated" increased with 6.8% but the correctly classified increased with an additional 14%.

BACKGROUND

Traditional pathological staging systems are still the most important tool for therapeutic decision making in colorectal cancer. However, pathological variables are only moderate indicators of outcome and therapy response. Twenty-five percent of stage II colorectal cancer patients (CRC) have recurrence of disease within 5 years. Current research focuses on the identification of this high risk group within the stage II CRC patients who would benefit from additional therapy. The Quasar collaborative group et al (1) reported a small benefit (3.6%) for chemotherapy (CT) treatment (fluorouracil and folinic acid) compared to observation within stage II CRC patients (1,2). This percentage is below the accepted level of 5% and therefore CT for the entire stage II groups is not advised.

Additional parameters of CRC, e.g. microsatelite-instability (MSI-high), have become of greater importance. MSI-high patients have been reported in several studies to have better prognosis compared to MSI-low.

Former studies have shown that a high intra-tumor stroma percentage predicts for CC patients with worse prognosis (3-5) and we postulated those patients would benefit from additional therapy. The intra-tumor stromal parameter has also been evaluated for esophageal and breast cancer and found to be an independent prognostic factor (6,7). For breast cancer the intra-tumor stromal percentage showed to be of additional predictive value for systemic therapy. The importance of intra-tumor stromal percentage and its use in therapy selection should be further examined.

Despite the frequency of colon cancer, the cellular and molecular characteristics of the target cells for oncological transformation and tumor-initiation at the primary site and distant metastasis is largely unknown. It is becoming increasingly clear that metastases develop when distant organs are seeded with this subpopulation of cancer cells with a stem/progenitor phenotype that arise from the primary tumor. The stroma is not an innocent bystander, but actively involved in formation and progression of malignant tumors. We hypothesize that disruption of these tumor-stroma interactions will inhibit or help to eliminate tumor progression and metastasis.

The current study presents a validation of our previous findings in colon cancer patients in a large independent series, the VICTOR trial (8,9). This trial was initially designed to monitor recurrence prevention by VIOXX in stage II-III CRC patients after potentially curative therapy.

METHODS

Patients

Tissue samples were collected within the study population of the VICTOR trial (8,9). Patients entering the VICTOR trial had undergone complete potential curative treatment including surgery alone or surgery plus radiation and/or chemotherapy within 12 weeks before entering the study. Inclusion criteria were: histologically proven Dukes B (Stage II; T3 or T4, N0, M0) or Dukes C (Stage III: any T, N1 or N2, M0) without gross or microscopically evidence of residual disease. Patients were randomized in a double blind design to receive rofecoxib or placebo for 2 or 5 years. They were recruited in 151 hospitals in the United Kingdom. For detailed trial design see Pendlebury et al. (9).

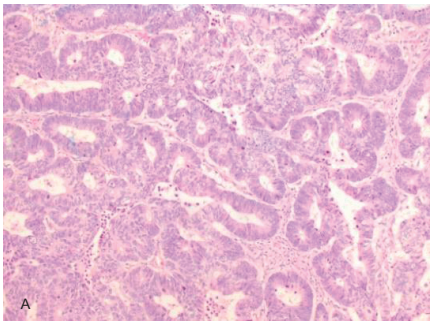
Initially the study was to have been completed in 2012 and aimed to recruit 7000 patients. Unfortunately the trial was closed to recruitment on 30 September 2004. Due to cardiovascular adverse effects of rofecoxib reported in the APPROVe trial (10-12) all patients were taken off the study drug. All randomized patients continued to be followed-up conform protocol. Kerr et al. describe no significant difference in mortality between patients with and without cardiovascular events within the VICTOR trial (13). Thus it may be expected that this does not influence OS in our analysis.

Histopathological scoring

Tissue samples consisting of 5µm Haematoxylin and Eosin (H&E) stained sections from the most invasive part of the primary tumor were used for analysis using conventional microscopy. The invasive front was chosen from the tissue block the pathologist selects as most invasive part and uses to determine the T-status. The most invasive tumor area on each slide was selected using a 2.5x or 5x objective. A part of the sample was selected where both tumor and stromal tissue were available using a 10x objective. Tumor cells must be present at all borders of the image field (north-east-south-west) (Figure 1). When mucinous tissue was present within a field that matched our scoring criteria, the mucinous tissue was visually excluded for the scoring. Two investigators (WM, GvP) estimated the stromal percentage in a blinded manner. In case of an inconclusive score a third observer was decisive (VS). Scoring percentages were given per tenfold (10, 20, 30% etc.) per image-field. For statistical analysis stromal ratio groups were divided into 'stroma-high (>50%)' and 'stroma-low (≤50%)' as determined a priori to have maximum discriminative power (4).

MSI status

For additional analyses MSI status was determined using initially 3 Bethesda microsatellites (Bat25, Bat26 and D2S123). Tumours with two unstable markers were classified



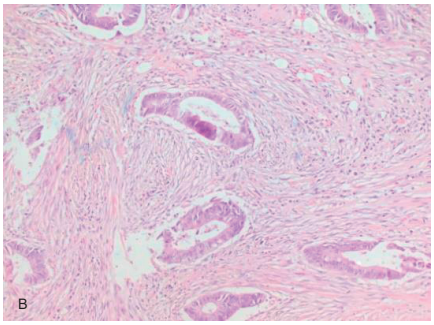


Figure 1. Haematoxylin and Eosin (H&E) stained 5 µm paraffin sections examined of the most invasive part of primary colon tumors. a) Stroma Low (20%) / b) Stroma High (80%)

as MSI and tumours without any unstable marker as MSS. Tumours with one single unstable marker were further analysed with the Bethesda marker D5S346 and the mononucleotide Bat40, which has been proven to be very useful for MSI identification (14). These tumours were classified as MSI if one of these two markers also displayed instability, otherwise they were classified as MSS.

Statistical analysis

Statistical analysis was performed using SPSS software version 17.0. Overall-Survival (OS) was defined as the time period between the randomization date and the date of death from any cause or the date of the last follow-up. Disease free survival (DFS) was defined as the time between the randomization date and the date of death or the date of first loco-regional or distant recurrence. If no recurrence occurred DFS was calculated as the time period until the date of last follow-up (15). Unfortunately no data were available on new primary tumors. 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 hazard model was used to determine the Hazard Ratio (HR) of explanatory variables for OS and DFS. MSI statistical analysis was performed using STATA 11.2.

RESULTS

Patients

In the VICTOR trial a total of 2434 patients were recruited between 2002 and 2004. A total of 959 histological samples were obtained from the participating clinics. Some of the samples were of poor histological quality and therefore excluded (N=20). After scoring all samples for the stromal parameter, additional patient information was collected. Due to the fact that most rectal cancer patients receive radiotherapy (RT) and

the known effect of RT on stromal formation in tissue we excluded all rectal cancer patients (N= 229). The stromal study cohort thus comprised of 710 patients.

Study population

Since only a part of the total study population was included for stromal analysis we compared our study population with the total VICTOR population. Between both groups no statistically significant differences were seen in gender, age, stage distribution, tumor localization, chemotherapeutic treatment or study-treatment arm (Rofecoxib/Placebo) (Table 1). Only a small difference in length of follow-up (FUP) was seen; total population mean FUP 52.1 (0-84.2) months compared to 55.4 (0-84.9) within the stromal study group ($p<0.0001$). Additionally no differences in number of deaths or recurrences were seen.

As can be found in Table 1 the stromal study consists of 438 men and 272 women, with a mean age of 65 years (range 25-86 years). Since patients had to first complete primary curative treatment, 61.0% (433) of them received adjuvant chemotherapy (CT) before randomization. After randomization 354 patients received rofecoxib and 356 were in the placebo treatment group. A total of 368 patients were stage II and 342 stage III (Supplementary Table S1).

Table 1. Comparison patient characteristics study and total population.

	Total study population, *N* = 1063	Stromal study population, *N* = 710
Males	646 (60.8%)	438 (61.7%)
Females	417 (39.2%)	272 (38.3%)
Stage II	526 (49.5%)	368 (51.8%)
Stage III	537 (50.5%)	342 (48.2%)
Colon	955 (89.8%)	637 (89.7%)
Rectosigmoid	108 (10.2%)	73 (10.3%)
Chemotherapy	687 (64.6%)	433 (61.0%)
No chemotherapy	376 (35.4%)	277 (39.0%)
Rofecoxib	534 (50.2%)	354 (49.9%)
Placebo	529 (49.8%)	356 (50.1%)
Age	64.1 (29–89)	64.8 (25–86)

Scoring stroma percentage

In 676 (95.2%) cases observers agreed on classification. Only in 34 (4.8%) cases there was no agreement between the observers; in those cases a third observer was decisive. Cohen's kappa coefficient revealed an almost perfect agreement in classification (Kappa = 0.89) (Figure 1).

Survival analysis

Out of 710 analyzed samples 207 (29.2%) were scored as stroma-high and 503 (70.8%) as stroma-low. In the stroma-high population the five year survival rate for OS was 69.0% versus 83.4% within the stroma-low population. For the DFS the five year survival rates for stroma-high and stroma-low were 58.6% versus 77.3% respectively. OS and DFS within the stroma-high group were as expected significantly lower than in the stroma-low group (OS p<0.0001, HR=1.96 (95%CI:1.41 to 2.74); DFS p<0.0001, HR=2.15 (95% CI:1.61 to 2.86)) (Figure 2). In uni,- and multivariate analysis, after adjusting for age, sex, stage, chemotherapy, tumor site, stroma percentage, vioxx treatment and MSI status, the tumor-stroma ratio was an independent prognostic factor for both OS (p = 0.002, HR 1.7 (95%CI: 1.2 to 2.4)) and DFS (p<0.001, HR 1.9 (95%CI: 1.4 to 2.6)) (Table 2). Because left and right sited tumors are known to have a different prognosis, a uni,- and multivariate analysis is repeated with this subdivision (Table 3). Descending colon and sigmoid were considered left sited and caecum, ascending colon, hepatic flexure, transverse colon and splenic flexure as right sited tumors. Unfortunately additional pathological information for 72 patients is lacking (in these cases site is classified as colon without further specifications). For this reason the analysis is performed for both total population and this subset of patients with more specific tumor-site status.

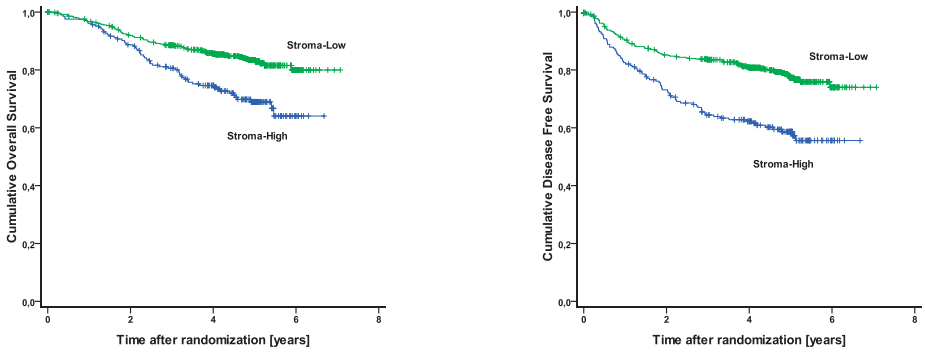


Figure 2. Kaplan-Meier survival curves of overall survival and disease free survival of stroma-high versus stroma-low in the total patient population (stage II and III) N=710 (OS p<0.0001, HR=1.96 (95%CI:1.41 to 2.74); DFS p<0.0001, HR=2.15 (95%CI:1.61 to 2.86)).

To account for systemic therapy effects the tumor stroma ratio was analyzed in a subgroup of patients treated with and without chemotherapy. The traditional pathological staging system (15) was used in combination with the ASCO criteria (16) to categorize patients as high risk or low risk within the stage II and III group. Patients with high risk are considered for adjuvant chemotherapy. In our study group 433 patients

received CT. Although this decision was made before randomization we assessed the stroma value of the high and low risk patients within our analysis. From all patients receiving CT, OS and DFS between stroma-high and stroma-low differed significantly

Table 2. Univariate & Multivariate analysis including age, sex, stage, chemotherapy, tumor site, stroma percentage, vioxx treatment and MSI status OS and DFS of total study population N=710.

	Univariate analysis						Multivariate analysis					
	Overall survival			Disease-free survival			Overall survival			Disease-free survival		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age												
<75 years	1		**0.005**	1		0.08	1		**0.001**			
≥75 years	1.71	1.18–2.49		1.35	0.96–1.90		1.88	1.28–2.77				
Sex												
Male	1		0.20	1		0.09						
Female	0.80	0.56–1.13		0.77	0.56–1.04							
Stage												
II	1		**<0.001**	1		**<0.001**	1		**<0.001**	1		**<0.001**
III	2.71	1.89–3.88		2.36	1.75–3.19		2.77	1.67–4.59		2.41	1.58–3.69	
Chemotherapy												
No chemotherapy	1		**0.001**	1		**<0.001**	1		0.75	1		0.45
Chemotherapy	1.88	1.30–2.73		1.78	1.30–2.45		0.92	0.53–1.57		0.84	0.54–1.32	
Site												
Colon	1		0.30	1		0.24						
Rectosigmoid	1.30	0.79–2.13		1.29	0.84–1.98							
Stroma												
Stroma-low	1		**<0.001**	1		**<0.001**	1		**0.002**	1		**<0.001**
Stroma-high	1.96	1.40–2.74		2.15	1.60–2.90		1.71	1.22–2.41		1.95	1.45–2.61	
VIOXX												
No VIOXX	1		0.58	1		0.50						
Vioxx	1.10	0.79–1.53		0.87	0.65–1.15							
MSI status												
MSS	1		0.66	1		0.09						
MSI	0.89	0.55–1.45		0.67	0.42–1.06							

Table 3. Univariate & Multivariate analysis including age, sex, stage, chemotherapy, tumor site, stroma percentage, vioxx treatment and MSI status OS and DFS of subpopulation with additional pathological information of tumor site N=638.

	Univariate analysis						Multivariate analysis					
	Overall survival			Disease-free survival			Overall survival			Disease-free survival		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age												
<75 years	1		**0.003**	1		**0.050**	1		**0.001**	1		0.014
≥75 years	1.79	1.22–2.62		1.41	1.00–1.99		1.98	1.34–2.93		1.56	1.10–2.23	
Sex												
Male	1		0.36	1		0.25						
Female	0.84	0.59–1.21		0.83	0.61–1.14							
Stage												
II	1		**<0.001**	1		**<0.001**	1		**<0.001**	1		**<0.001**
III	2.69	1.86–3.90		2.38	1.75–3.25		3.07	1.83–5.15		2.40	1.55–3.68	
Chemotherapy												
No chemotherapy	1		**0.002**	1		**0.001**	1		0.33	1		0.74
Chemotherapy	1.83	1.25–2.67		1.75	1.26–2.42		0.92	0.54–1.60		0.92	0.58–1.47	
Site												
Left	1		***0.051***	1		0.24	1		**0.004**			
Right	1.41	1.00–1.99		1.20	0.89–1.61		1.66	1.17–2.36				
Stroma												
Stroma-low	1		**<0.001**	1		**<0.001**	1		**0.008**	1		**<0.001**
Stroma-high	1.86	1.31–2.63		2.09	1.55–2.82		1.66	1.17–2.29		1.88	1.38–2.54	
VIOXX												
No VIOXX	1		0.59	1		0.34						
VIOXX	1.10	0.78–1.55		0.87	0.64–1.16							
MSI status												
MSS	1		0.66	1		0.09						
MSI	0.89	0.55–1.45		0.67	0.42–1.06							

(OS p=0.002, HR=1.85 (95%CI: 1.25 to 2.72); DFS p<0.0001, HR=2.03 (95%CI: 1.45 to 2.86)) with 5 year survival rates of stroma-high: OS 65.5%, DFS 54.5% compared to stroma-low: OS 80.8%, DFS 74.2%. Within the 'low-risk' group of patients not receiving CT, there was no significant difference of OS and DFS comparing stroma values (OS p=0.210, HR=1.58 (95%CI:0.77 to 3.26); DFS p=0.048, HR=1.81 (95%CI:0.99 to 3.28)) (Supplementary Figure S1 and S2).

From 368 stage II CC patients analyzed, 83 were scored as stroma-high and 285 as stroma-low. The differences for OS and DFS between stroma-high and stroma-low were OS p=0.034, HR=1.95 (95%CI:1.04 to 3.65); DFS p=0.0005, HR=2.04 (95%CI:1.23 to 3.40). Five year survival rates for overall and disease free survival time respectively were 79.8% versus 89.1% and 71.1% versus 83.3% for stroma-high versus stroma-low (Supplementary Figure S3).

The stage III CC group consisted of 342 patients of which 124 were scored stroma-high and 218 as stroma-low. There were significant differences in survival time for this group of patients when comparing stroma-high and stroma-low (OS p=0.019, HR=1.61 (95%CI: 1.07 to 2.39); DFS p<0.0001, HR=1.86 (95%CI: 1.30 to 2.64)). Five year overall and disease free survival rates for the stroma-high group versus the stroma-low group were 61.7% versus 76.1% and 50.2% versus 69.4% respectively (Supplementary Figure S4).

Relation between MSI status and intra-tumour stroma proportion

To evaluate whether there could be a relation between MSI status and the stroma percentage additional analyses were performed. Within our study population (N=710) MSI data of 662 patients were available. Within this group 558 patients were classified as MSS and 104 as MSI. Within the MSS group 178 (31.9%) are stroma-high and 389 (69.7%) stroma low. The MSI group consists of 20 (19.2%) stroma-high and 84 (80.7%) stroma-low. Stroma and MSI were found to be associated; Chi-square p=0.010.

Correlation of T stage and N stage to the intra-tumour stroma proportion

The relation between TNM stage and intra-tumour stroma patients is evaluated. TNM data of 661 patients were available. Because all patients included in this study are stage II or stage III patients, only T and N stage were considered. Therefore the stroma percentages within the T stage and N stage groups were compared with a chi-squared test. Both the T and the N status were significantly related to the stroma percentage (T-status p <0.0001 and N-status p=0.005). All T1 (n=4) patients were stroma-low. 96.2% of the T2 (n=26) patients were stroma-low. In the T3 group (n=460) this per-

centage decreased to 74.8% and in the T4 group (n=171) it was only 55%. For the N status the stroma low percentage in the N0 group (n=348) was 76.1%, in the N1 group (n=210) 64.3% and in the N2 group (n=67) 65% (Figure 3).

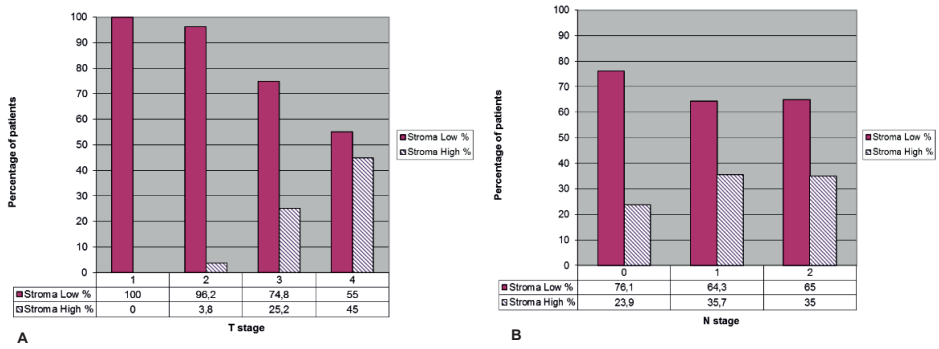


Figure 3. Correlation of T stage and N stage to the intra-tumour stroma proportion.

Comparing the intra-tumor stroma ratio with the ASCO high risk criteria

To identify high risk stage II CC patients that might benefit from adjuvant CT, ASCO proposed several high risk criteria for clinical implementation. These criteria include T4 tumor stage, a lymph node yield less than 10 nodes in the resection specimen, poor tumor differentiation, vascular invasion or perforation of the bowel wall at presentation. We compared the efficiency of these ASCO criteria in the identification of high risk patients to our stroma parameter. For this we used a subset of our study population consisting of 256 Stage II CC patients that did not receive any adjuvant therapy. Based on the ASCO criteria 119 patients were classified as high risk. With the addition of the stroma parameter to the ASCO criteria 140 patients were classified as high risk. The addition of the stroma parameter improved the false negative rate of ASCO criteria and correctly identified 14% (N=4) more patients (i.e. of patients that were not classified as high risk by the ASCO criteria but indeed developed a distant metastasis or died due to CC in the follow up period). As a conclusion the rate of "undertreated" patients based on the ASCO criteria dropped from 5.9% to 4.3% and the correctly classified increased with an additional 14% when using the ASCO-stroma parameter combination.

DISCUSSION

Our study confirms previous findings that the intra-tumor stroma percentage is an independent factor for prognosis of CC patients. Patients with a high intra-tumor stroma percentage have a significantly worse prognosis than those with a low stroma percentage, with a consistent hazard ratio of about two. In multivariate analysis, even after correction for TNM stage, the tumor-stroma ratio remained an independent prognostic factor for both OS ($p = 0.002$, HR 1.7 (95%CI: 1.2 to 2.4)) and DFS ($p<0.001$, HR 1.9 (95%CI: 1.4 to 2.6)).

To our knowledge we are the first group to describe the intra-tumor stroma ratio as a independent prognostic parameter (3,4). This method was applied for automation by West et al. (5) and they validated our findings with similar results: (HR)2.087, 95%CI: 1.08 to 4.00, P=0.024 using a cut-off value of 47%.

Our study suggests that an increased amount of stromal involvement, even if it is detected in only a small part of the total tumor mass, can be linked to an unfavorable prognosis, independent of other prognostic parameters. Possibly, this particular part of the tumor has obtained the capability to orchestrate its direct environment to facilitate its invasive and metastatic behavior.

Currently, next to traditional histopathological staging, MSI status is advised as an indicator for therapy choice and possible predictor for prognosis (17-21). In this study MSI status showed no significant differences in OS and DFS. Stroma and MSI were found to be associated; Chi-square p=0.010. As expected in relation to survival, within the MSI group the number of stroma-low patients was higher (80.7% vs. 19.2%). The same was seen in the MSS group, this group consisted of a higher number of stroma-high patients (69.7% vs. 31.9%).

Our high intra-observer agreement with a kappa value of 0.89 in this study and scoring in previous studies indicates that the intra-tumor stroma proportion is a highly reproducible measurement. The previously published stromal study in a CC patient group showed kappa values between three different observers varying between 0.60 and 0.70 (concordance 93%) (3,4). For esophageal cancer and breast cancer Cohen's kappa coefficient for two observers was respectively 0.86 and 0.85 (6,7).

The relation between TNM stage and intra-tumour stroma patients is evaluated. It shows that with the increase of T and N stage the number of stroma-high patients grows. This is as expected.

ASCO proposed guidelines to identify high risk stage II CC patients that might benefit from adjuvant CT (16). Our study showed that with adding the stroma-parameter to the ASCO criteria the rate of "undertreated" patients dropped from 5.9% to 4.3% and the correctly classified increased with an additional 14% when using the ASCO-stroma parameter combination. This comparison is a good parameter to measure how addition of the stroma parameter can improve current high risk stratification methods. However to compare the efficiency of adding the stroma parameter to the ASCO criteria should ideal be tested in a prospective study instead of a subset of untreated stage II CC patients like in this case.

A secondary aim of stromal analysis within the VICTOR trial was to investigate association with therapy response. Therefore the different treatment arms were compared for OS and DFS. There is a statistical drawback with this analysis. Within the study population 'high-risk' patients were selectively treated following current treatment protocols with CT before randomization and 'low risk' patients did not receive CT.

Within the low-risk treated patients the stroma-parameter showed no difference. Although we have found in former studies that a small number of patients with low-risk have a stroma-high tumor, probably in this study the number is too low to reach statistical significance.

In conclusion, we found the stroma parameter to be a simple and reproducible prognostic parameter which may indicate important differences in biology. It is remarkable that a simple cell based parameter using conventional microscopy can possess such a high predictive power without any additional costs. This parameter does not seem to be limited to CC but is also relevant as new prognostic factor for esophageal and breast cancer.

In this manuscript we validated the stroma parameter to select patients at risk for death or recurrence of disease for additional therapy. This parameter is to be expected to be used in clinical practice for better risk-classification and should therefore be considered for implementation in standard pathology reports together with the MSI status in addition to the current TNM classification.

SUPPLEMENTARY TABLE & FIGURES

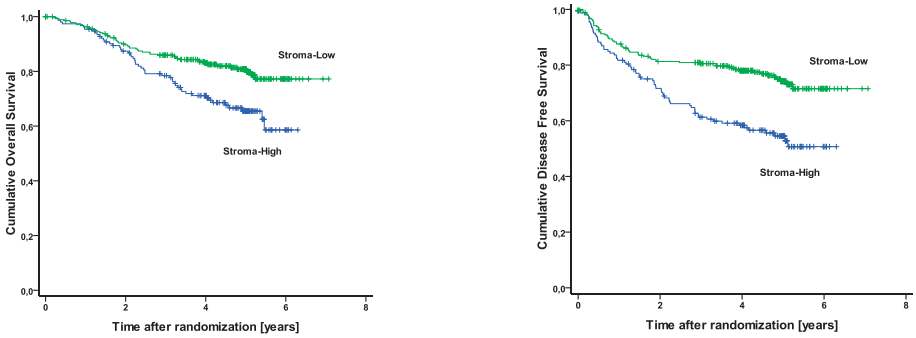


Figure S1. Kaplan-Meier survival curves of overall survival and disease free survival of stroma-high versus stroma-low in all patients receiving CT N=433 (OS p=0.002, HR=1.85 (95%CI: 1.25 to 2.72); DFS p<0.0001, HR=2.03 (95%CI: 1.45 to 2.86)).

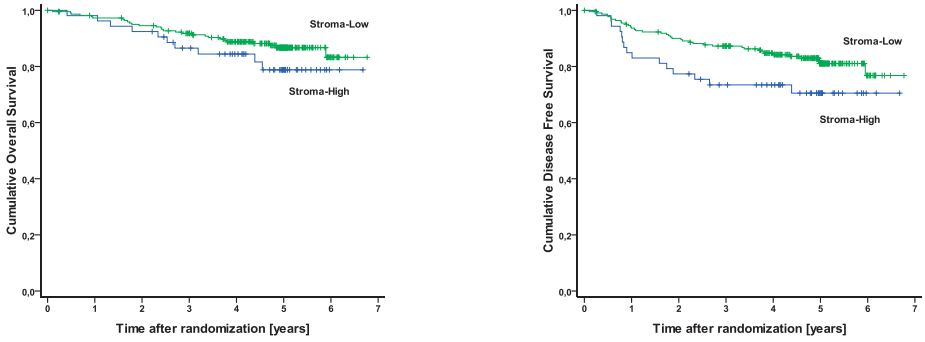


Figure S2. Kaplan-Meier survival curves of overall survival and disease free survival of stroma-high versus stroma-low in all patients not receiving CT N=277 (OS p=0.210, HR=1.58 (95%CI:0.77 to 3.26); DFS p=0.048, HR=1.81 (95%CI:0.99 to 3.28)).

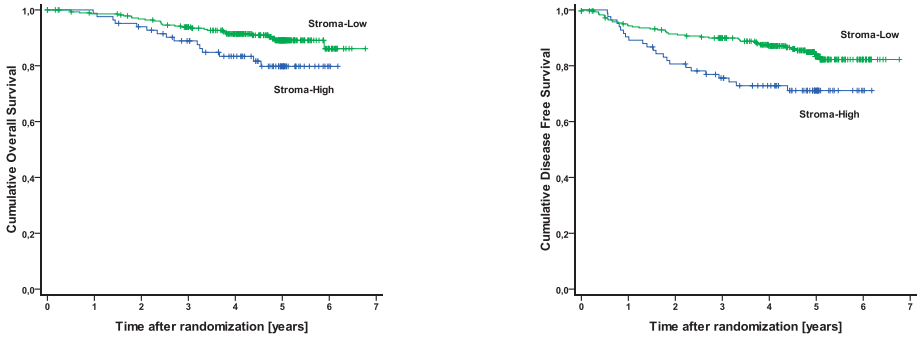


Figure S3. Kaplan-Meier survival curves of overall survival and disease free survival of stroma-high versus stroma-low in stage II CC patients N=368 (OS p=0.034, HR=1.95 (95%CI:1.04 to 3.65); DFS p=0.0005, HR=2.04 (95%CI:1.23 to 3.40)).

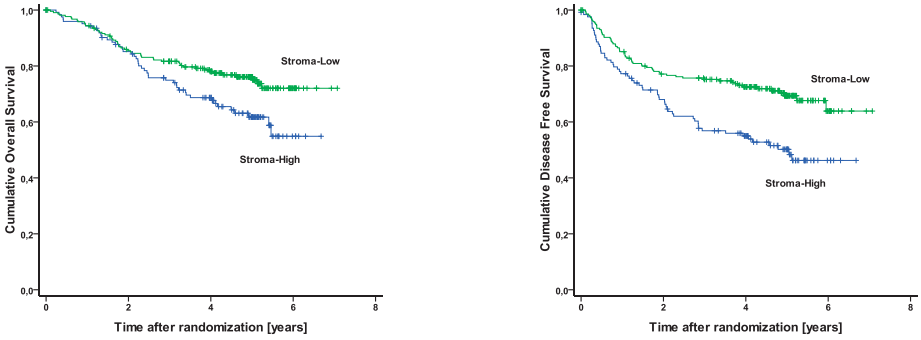


Figure S4. Kaplan-Meier survival curves of overall survival and disease free survival of stroma-high versus stroma-low in stage III CC patients N=342 (OS p=0.019, HR=1.61 (95%CI: 1.07 to 2.39); DFS p<0.0001, HR=1.86 (95%CI: 1.30 to 2.64)).

Table S1. Patient characteristics stroma-high versus stroma-low group. Chi-squared p<0.0001.

	Stroma-high	Stroma-low	Total
Stage II	**83 (22.6%)**	**285 (77.4%)**	**368**
Stage III	**124 (36.3%)**	**218 (63.7%)**	**342**
Total	**207 (28.2%)**	**503 (70.8%)**	**710**

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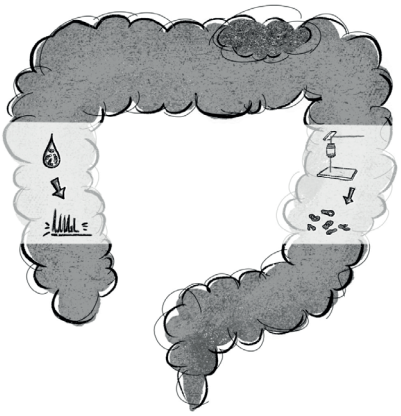

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The value of additional bevacizumab in patients with high-risk stroma-high colon cancer. A study within the QUASAR2 trial, an open-label randomized phase 3 trial.

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Journal of Surgical Oncology 2018 Apr;117(5):1043-1048.

ABSTRACT

Introduction: Patients with a high stroma percentage within the primary tumor have a poor prognosis. In this study we investigate whether anti-angiogenic therapy might improve survival of patients with a stroma-high profile with potentially increased angiogenesis.

Materials and Methods: Tissue samples of the primary tumor of 965 colon cancer patients participating in the QUASAR2 trial were analyzed for tumor-stroma ratio (TSR). Stroma-high (>50%) and stroma-low (≤50%) groups were evaluated with respect to survival.

Results: Disease free survival (DFS) was significantly lower in the stroma-high group (HR 1.53, 95%CI 1.19-1.95, p=0.001). No difference in DFS was seen with respect to treatment with capecitabine alone (CAP) or capecitabine with bevacizumab (CAPBEV) (Stroma-high HR 1.00, 95%CI 0.69-1.46, p=0.996; stroma-low HR 1.02, 95%CI 0.75-1.41, p=0.883). A significant difference in survival was seen comparing groups with or without vascular invasion (DFS p<0.001). A correlation between vascular invasion and stroma-high was seen (χ2-test p=0.043).

Discussion and Conclusions: The TSR confirmed to be a strong prognosticator for disease-free survival in a selected high-risk patient population. No benefit was found in response to treatment with bevacizumab when stratified for TSR. TSR showed to have an additional prognostic value in patients with vascular invasion present in the primary tumor.

INTRODUCTION

The tumor-stroma ratio (TSR) in colon cancer (CC) patients has previously been reported by our group and others as a strong independent prognostic parameter. Patients with CC and a high stroma percentage within the primary tumor have a poor prognosis (1-5).

The knowledge of interactions between cancer cells and their tumor microenvironment (TME) and its associated stromal cells is of increasing importance. There is an interaction between non-malignant cells of the microenvironment and malignant cells with growth factors and chemokines that stimulate cancer cell growth, migration and invasion (6). The tumor-stroma itself has been shown to play an important role in tumor formation and progression (7). The tumor-stroma environment contains multiple different cells including (cancer-associated) fibroblasts, angiogenic vascular cells and infiltrating immune cells (8). One of the hallmarks of tumor progression is angiogenesis which the tumor stroma facilitates. When changes occur in the TME, stroma can modulate cancer development and progression (9). Although some aspects of stroma are understood quite well, in particular the contribution of tumor angiogenesis and remodeled extracellular matrix (ECM), it becomes more evident that stromal cells play a much larger role in tumor growth and progression than previously thought (6).

The prognostic value of the TSR has been previously shown, but examining the TSR and its use in therapy selection is a promising new approach. Personalized therapy based on the characteristics of the individual tumor could improve survival and decrease adverse effects induced by unnecessary therapy. The stromal environment contributes to tumor angiogenesis, which supplies the oxygen and nutrients needed for tumor growth and progression (10). Anti-angiogenic therapy, for example with bevacizumab, a monoclonal antibody against vascular-endothelial growth factor, can therefore play an important role in treating patients with increased angiogenesis. Therapy targeting the TME could make a difference in survival, especially for the stroma-high group. This patient group shows a worse survival compared to stroma-low patients and recent literature indicates the resistance of stroma-high patients to current standard chemotherapy regimens (11).

In this present study we investigate the additional value of bevacizumab to standard chemotherapy for stroma-high patients. Furthermore, the relation between the tumor-stroma ratio and the presence of vascular invasion is analyzed.

MATERIAL AND METHODS

Patients

Tissue samples of patients with colon cancer were obtained from the study population of the Quick and Simple and Reliable trial (QUASAR2)(9), a phase III randomized trial of adjuvant capecitabine (CAP) ± bevacizumab (BEV) after complete surgical resection of high-risk stage II and stage III colorectal cancer. Inclusion criteria were histologically proven stage II (stage T4, lymphatic invasion, vascular invasion, peritoneal involvement, poor differentiation, obstruction and perforation of the primary tumor during the pre-operative period and T3 as long as they also have one of the previous listed poor prognostic features) and stage III (any T, N+, M0)(12). QUASAR2 samples were recruited in 123 UK and 61 non-UK participating hospitals. For detailed trial design see http://www.oncology.ox.ac.uk/trial/quasar-2.

Histopathological scoring

Tissue samples consisting of 5 µm Haematoxylin and Eosin (H&E) stained formalin-fixed paraffin-embedded sections from the most invasive part of the primary tumor were used for TSR scoring using conventional microscopy. TSR was defined as the percentage intra-tumor stroma tissue relative to the neoplastic cell component. The protocol for TSR scoring has been described previously (1, 4). In short, the area with the highest amount of stroma on each slide was selected using a 2.5x or 5x objective. Using a 10x objective areas where tumor cells are present at all borders of the image field were scored. Scoring percentages were given per tenfold (10, 20, 30% etc.) per image-field. When mucinous tissue was present within a field that matched our scoring criteria, the mucinous tissue was visually excluded for the scoring. Two investigators (GvP,AH) estimated the stromal percentage in a blinded manner. In case of discrepancy slides were reviewed to reach consensus. In case no consensus could be reached a third observer (V. Smit) was decisive. For statistical analysis stromal ratio groups were divided into stroma-high (>50%) and stroma-low (≤50%) (Figure 1).

Statistical analysis

Statistical analysis was performed using SPSS software version 23.0. Disease-free survival (DFS) was defined as the time from randomization until confirmation of relapse or death of any cause. If no recurrence occurred DFS was calculated as the time period until the date of last follow-up. Inter-observer variability was analyzed using the Cohen's kappa coefficient. 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 hazard model was used to determine the Hazard Ratio (HR) of explanatory variables for DFS.

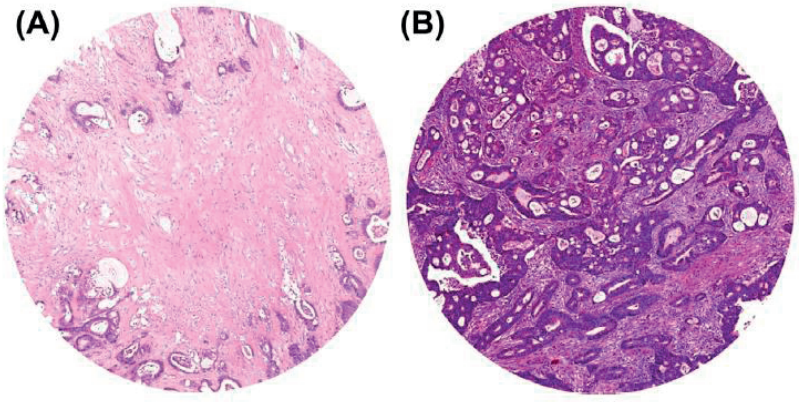

Figure 1. Examples of stroma-high (a) and stroma-low (b) haematoxylin and eosin (H&E) stained paraffin sections at the most invasive part of primary colon cancers (200x magnification).

RESULTS

Patients

In the QUASAR2 trial a total of 1389 patients with colorectal cancer were recruited between 2005 and 2010. A total of 1069 histological samples were obtained from the participating clinics. After scoring all samples for TSR, additional patient information was collected. Rectal cancer patients were excluded from the analysis (N=76) due to the fact that most of them received pre-operative radiotherapy (RT) with known effect of stromal formation. Of 15 samples it was not possible to score a proper TSR due to inferior histological quality. Another 13 patients were excluded due to tumor location or additional pathology information (N=3 small bowel or appendix, N=7 double tumor, N=2 pM1, N=1 stage I). As shown in table 1 the final TSR study cohort comprised of 965 patients (356 stage II, 609 stage III). The study population consisted of 548 men and 417 women, with a mean age of 63.8 years (SD 9.8) years. Within this group 459 patients received CAP and 506 received CAPBEV. Vascular invasion was present in 357 patients (37.0%), in 568 patients there was no vascular invasion (58.9%) and of 40 patients this data was missing (4.1%).

Tumor-stroma ratio

Of in total 965 patients, 323 (33.5%) patients were classified with a stroma-high tumor and 642 (66.5%) with a stroma-low tumor. Cohen's Kappa coefficient revealed a good agreement in classification (k=0.73, 87% concordance). From 357 patients with vascular invasion 135 (37.8%) were stroma-high and 222 (62.2%) were stroma-low. Within the group without vascular invasion (N=568) the division stroma-high versus stroma-low was 178 (31.3%) versus 390 (68.7%), respectively.

Survival analysis

In the stroma-high population the five year survival rate for DFS was 65% versus 75% within the stroma-low population. As expected, the DFS within the stroma-high group was significantly lower compared to the stroma-low group (HR=1.53 (95% CI: 1.19 – 1.95, p=0.001))(Figure 2). In multivariate analysis, after adjusting for age, sex, stage, lymphatic invasion and vascular invasion, the TSR was an independent prognostic factor (HR=1.52 (95%CI: 1.18 – 1.96, p=0.001)).

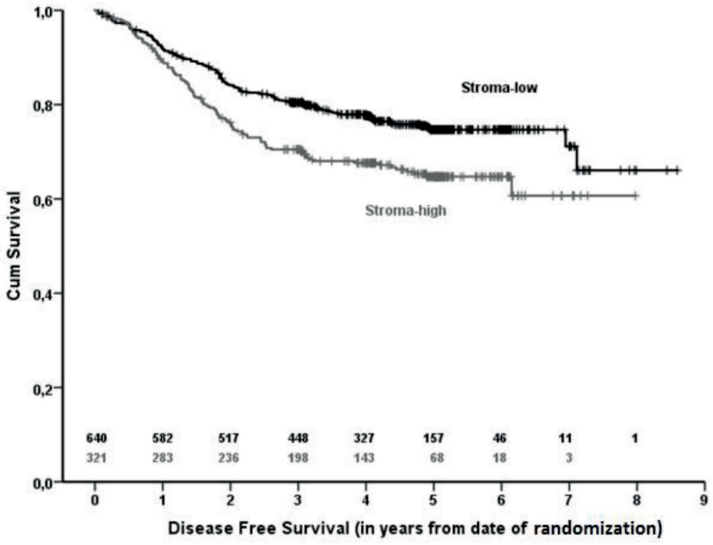

Figure 2. Kaplan-Meier disease free survival curve of the total patient group stratified for the tumor-stroma ratio.

Because of our hypothesis that stroma-high patients might benefit from bevacizumab due to its potential anti-angiogenic effect, we compared the results of therapy for this group of patients. No significant difference for stroma-high patients who received CAPBEV compared to those who were treated with CAP was observed (Stroma-high HR 1.00, 95%CI 0.69-1.46, p=0.996; stroma-low HR 1.02, 95%CI 0.75-1.41, p=0.883) (Figure 3).

Vascular invasion is a prognostic parameter for patients with colorectal cancer. To evaluate a possible interaction between vascular invasion and the TSR, survival times were compared. The DFS between patients with or without vascular invasion showed a significant difference (HR 1.64, 95%CI 1.28-2.10, p<0.001) with a shorter disease-free survival for patients with vascular invasion. Within this group with vascular invasion TSR could further subdivide for patients with worse survival (HR 1.44, 95%CI 1.01-2.06,p=0.041)(Figure 4). A correlation between the presence of a high amount of stroma and vascular invasion was observed (χ2-test p=0.043).

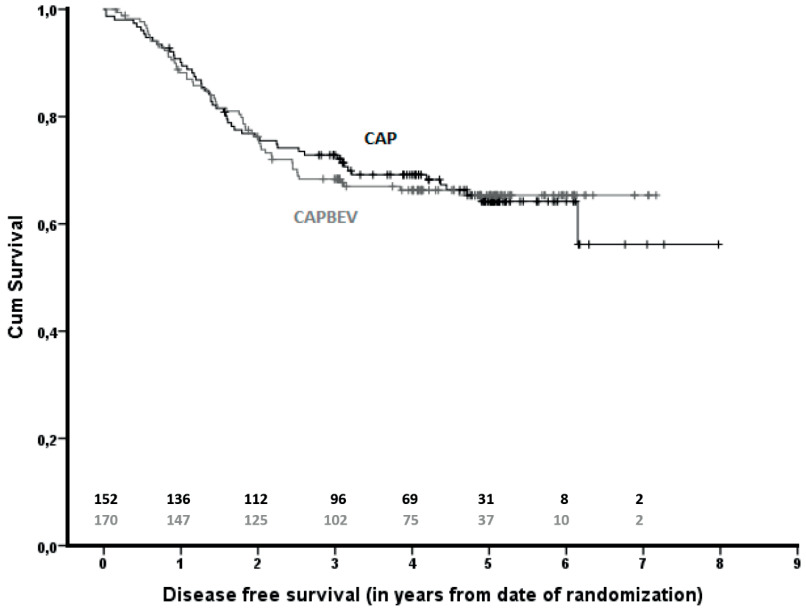

Figure 3. Kaplan-Meier disease free survival curve for the stroma-high patient group stratified for treatment.

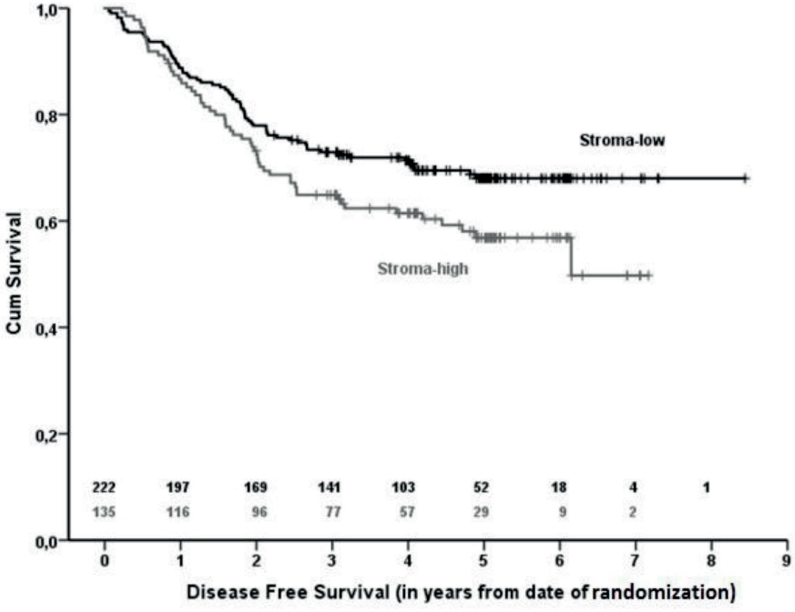

Figure 4. Subgroup analysis within patients positive for the presence of vascular invasion; Kaplan-Meier disease free survival curve stratified for tumor-stroma ratio.

DISCUSSION

Although our study population consisted of only high-risk patients, the TSR proved again to be a strong independent prognostic factor for CC patients. In addition, a worse survival for patients with vascular invasion was confirmed, which is known to be significantly related to reduced disease free and overall survival (13-15). The relation between patients with a stroma-high tumor and vascular invasion found in this study has not been described earlier. This correlation confirms the hypothesis of the important role angiogenesis plays in the stromal environment and the choice for therapy. Targeting the TME can make a difference in survival, especially for the subgroup of stroma-high patients.

The original study (12) did not show a benefit for the addition of bevacizumab for the total study population. In our study, analyzing subgroups of patients based on the pattern of stromal formation within the primary tumor, additional treatment with bevacizumab as anti-angiogenic therapy also did not improve the survival of stroma-high patients. Bevacizumab is a humanized monoclonal antibody which binds to VEGF, thereby prohibiting binding to VEGFR-1 and VEGFR-2. Carmeliet et al. described the complex role of inhibition of angiogenesis. Inhibition of a single target, for instance anti-VEGF therapy, could lead to upregulation of additional angiogenic factors (like PDGF). Combined treatment of anti-angionic agents could increase efficacy and may give the tumor(-microenvironment) less chance to escape from treatment (16). Multiple studies are further investigating the role of the TME and its stromal cells. Their relationship is fundamental for understanding tumor progression and therapeutic resistance. It has been recognized that the tumor-stroma influences drug uptake and sensitivity by different mechanisms. The tumor-stroma is for example involved in buffering the acidic tumor micro-milieu. During rapid tumor growth the TME becomes hypoxic. This induces the immigration of vessels into the tumor and also forces tumor cells to use alternate metabolic pathways creating an acidic microenvironment (7, 17). Moreover, the physical properties and composition of the TME can limit drug-uptake through a dysfunctional vasculature and increased interstitial fluid pressure (6, 7). The organization of the stromal matrix formation might also be an important factor for prediction of therapy response. Efficient organization of this matrix might increase the effective path of molecules towards the target cells (18). This might influence drug diffusion and treatment efficacy. A recent study confirms this hypothesis by describing tumors (of breast cancer patients) with stroma consisting of organized collagen showing a higher benefit from neo-adjuvant chemotherapy compared to tumors with disorganized stroma (19).

In this study, analyzing a pre-selected high-risk patient population with colon cancer, the TSR confirmed to be a strong prognosticator for disease-free survival. Furthermore, it proved to have an additional prognostic value in patients with vascular invasion present in the primary tumor. No benefit was found for the stroma-high group in response to treatment with bevacizumab. Further knowledge of the stromal composition might lead to new targeted treatment regimens focusing on patients with stroma-high and thus more aggressive tumors.

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Unravelling the tumor associated stroma in colon cancer patients using laser capture microdissection and reverse phase protein microarray.

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ABSTRACT

Introduction

The tumor microenvironment is an important target for cancer therapy. The prognostic value of the tumor-stroma ratio in colon cancer patients is well described. In order to evaluate the contribution of the underpinning signalling and molecular architecture of the tumor associated stroma to the aggressive stroma-high phenotype, we utilized laser capture microdissection coupled to broad-scale protein pathway activation mapping using reverse phase protein microarrays.

Material and Methods

Patients with histologically proven stage II and stage III colon cancer were selected from the LUMC database. Hematoxylin and Eosin (H&E) stained sections from the most invasive part of the primary tumor were scored for the tumor-stroma ratio. Reverse phase protein microarray was performed using micro-dissected material to generate multiplexed pathway profiling. For each sample, 58 endpoints were analysed.

Results

Comparison of the 58 endpoints in 30 colon cancer patients (15 stroma-high and 15 stroma-low) showed that phosphorylation of VEGFR-2 was significantly higher in the stroma-high group compared to the stroma-low group ($p=0.02$) and that ZAP70, eNOS and ICAM-1 was significantly lower in the stroma-high group ($p=0.01$, $p=0.04$ and $p=0.03$, respectively). Correlation analysis showed a major eNOS node with many interconnections in the tumor stroma within the stroma-low group.

Conclusion

This pilot study showed the potential presence of biochemical derangements in the tumor stroma of tumors from patients with aggressive colon (stroma-high) cancer with increased activation of VEGFR-2 and decreased activation of ZAP70, eNOS and ICAM-1. Focusing on the stroma-low group, there is a significantly higher expression of eNOS with many interconnections including ARPC2. These interconnections may contribute to the better behaviour of the stroma-low tumors.

INTRODUCTION

The tumor microenvironment, and especially the stroma surrounding the tumor cells, is an important target for cancer therapy. Tumor stroma plays an important role in tumorigenesis. From initiation to invasion and metastasis, tumor stroma interacts with both malignant and non-malignant cells.

Tumor stroma is composed of a mixture of cells, including immune cells, fibroblasts and endothelial cells, that are embedded in the proteins of the extracellular matrix (ECM). When the ECM interacts with the tumor cells, it will influence disease progression and metastatic capacity. One of the most important cell types of the tumor stroma is the activated form of fibroblasts, the so-called cancer-associated fibroblasts (CAFs). CAFs are involved in tumor progression and invasion. Stromal cells stimulate blood vessel formation and supply the tumor with growth factors, cytokines and metabolites [1]. This might explain the decreased survival time for patients with a tumor with high stromal content.

Tumor-stroma ratio (TSR) distinguishes between aggressive and non-aggressive tumors. The prognostic value of TSR is well described and validated [2-4]. Colon cancer patients with a high (>50%) amount of intratumor stroma have a poor prognosis compared to patients with a low amount (<=50%) [2-5]. The TSR is easily determined on conventional hematoxylin and eosin (H&E) stained tissue sections used for routine pathological investigation. Moreover, in breast and esophageal cancer, this prognostic parameter has also been validated in large patient series [6-10]. Furthermore, in other types of epithelial cancer (for example cervical, prostate, bladder, head/neck and lung cancer), the same prognostic value was found by different international research groups [11-17].

However, it is not yet entirely clear why TSR makes this distinction between aggressive and non-aggressive tumors since the underlying mechanism is still not fully understood. Nevertheless, we do know that tumor stroma plays an important role in tumor formation and progression [18]. A colon tumor with a high stromal content has a highly increased interaction between tumor and stromal cells. Specific molecular changes in colon cancer cells cause the recruitment and activation of surrounding stromal cells, which enables tumor progression by releasing soluble growth factors, metabolites and cytokines [18].

In order to evaluate the contribution of the underpinning signalling and molecular architecture of the tumor associated stroma to the aggressive stroma-high pheno-

type, we utilized laser capture microdissection (LCM) coupled to broad-scale protein pathway activation mapping using reverse phase protein microarrays (RPMA). This technique uses cellular enrichment of specific tissue cells via LCM for tissue biomarker discovery and selection criteria for personalized treatment [20-23]. RPMA is a high throughput multiplex proteomic platform. It has the ability to measure hundreds of analytes in a large number of samples with only a small amount of biological material [24-26]. By focusing on activation in terms of phosphorylation, next to kinase expression, this technique has been successful for signalling network analysis [27-30]. Such analysis could identify new stromal-based targeted information interesting for treatment options through the identification of activated pathways within the tumor stroma of patients with aggressive colon cancer.

MATERIAL & METHODS

Patients

Patients with histologically proven stage II and stage III colon cancer were selected from the Leiden University Medical Center (LUMC, The Netherlands) database. All patients underwent surgical resection of the primary tumor between 2001 and 2011, with or without adjuvant chemotherapy. Only patients whose fresh frozen tumor material was available were selected. Patients who received neo-adjuvant treatment were excluded.

Histopathological scoring

By using conventional microscopy, H&E stained sections from the most invasive part of the primary tumor were scored for the amount of stroma. TSR was defined as the percentage intra-tumor stroma tissue relative to the neoplastic cell component. The protocol for TSR scoring has been described previously [2, 31]. In short, the most invasive tumor area with the highest amount of stroma on each slide was selected using a 2.5x or 5x objective. Using a 10x objective areas where tumor cells are present at all borders of the image field were scored. Scoring percentages were given per tenfold (10, 20, 30% etc.) per image-field (Figure 1). When mucinous tissue was present within a field that matched our scoring criteria, the mucinous tissue was visually excluded for the scoring. Two investigators (GvP, AH) estimated the stromal percentage in a blinded manner. In case of discrepancy slides were reviewed to reach consensus. In case no consensus could be reached a third observer was decisive (WM). Patients were categorized into two groups; a stroma-high (>50% stroma) and a stroma-low (=<50% stroma) group.

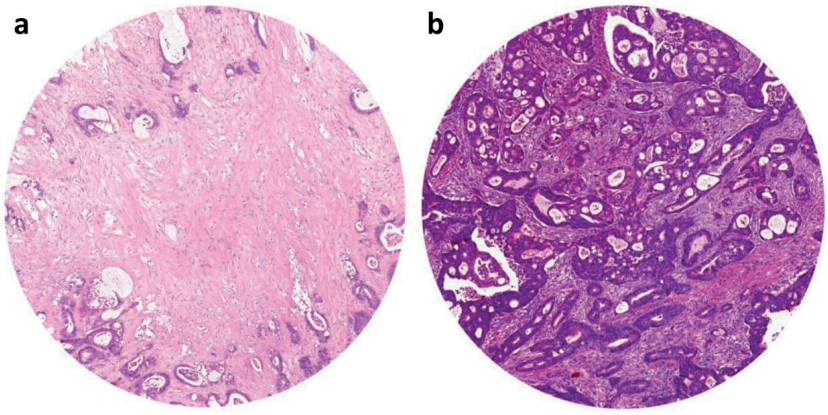


Figure 1. Examples of stroma-high (a) and stroma-low (b) haematoxylin and eosin (H&E) stained paraffin sections at the most invasive part of primary colon cancers (100x magnification).

Laser capture microdissection (LCM)

As described previously, highly enriched stromal cell subpopulations were obtained using Arcturus Veritas 704 LCM System (Arcturus, Mountain View, CA USA) [32, 33] (Figure 2). Stroma cells were captured when they were surrounded by tumor epithelium cells on all four corners of microscopic field with a magnification of 20x and the stroma was in direct contact with the external edge of the tumor. Lymphocyte agglomerates, when present, were not captured. Microdissected material was stored at -80°C and cell lysates were prepared for RPMA as previously described [28, 34].

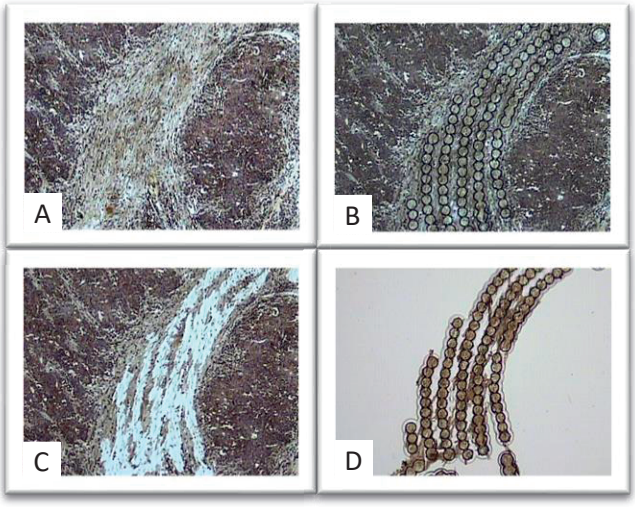


Figure 2. Laser capture images from tumor associated stroma cells before and after LCM.
A) Inter tumor stroma area microscopically selected. B) Selected tumor stroma tissue by laser capture. C) After laser capture. D) Removed stroma tissue for analysis on a nitrocellulose membrane.

Reverse phase protein microarray analysis (RPMA)

Cell lysates were printed in triplicate onto nitrocellulose coated slides Using a 2470 Aushon Arrayer (Aushon BioSystems, Billerica, MA, USA) along with standard curves for quality assurance (Grace BioLabs, Bend, OR, USA). A complete list of all 58 proteins and phosphoproteins measured in this study are listed in table 2. These analytes were chosen based on their involvement in key aspects of epithelial mesenchymal transition, ECM composition and remodeling, angiogenesis, inflammation and transcription. All antibodies used in these studies were validated by western blotting for single band specificity prior to use [35-38]. Immunostaining was performed as previously described [34]. Concisely, each slide was incubated with one primary antibody targeting the protein of interest. As secondary antibodies biotinylated goat anti-rabbit (1:7,500, Vector Laboratories Inc, Burlingame, CA) and rabbit anti-mouse (1:10, DakoCytomation, Carpinteria, CA, USA) IgG were used. Using a tyramide-based avidin/biotin amplification system (DakoCytomation, Carpinteria, CA, USA) coupled with Streptavidin conjugated IRDye 680 (LI-COR, Lincoln, NE, USA) signal amplification and detection were achieved. Total protein was measured following manufacturing instructions using a Sypro Ruby protein blot staining protocol (Molecular Probes, Eugene, OR, USA). With a Tecan PowerScanner (Tecan, Männedorf, Switzerland) images were acquired and analyzed with the MicroVigene software Version 5.1.0.0 (Vigenetech, Carlisle, MA, USA) [34].

Table 1. Patient characteristics

	Total		Stroma-low		Stroma-high		
	N = 30	%	N = 15	%	N = 15	%	P-value
Age (median in yrs [range])	67 [38-90]		70 [58-90]		64 [38-79]		0.47
Gender							
Female	14	47	8	53	6	40	0.46
Male	16	53	7	47	9	60	
Tumor Location							
Left-sided	14	47	7	47	7	47	1.00
Right-sided	16	53	8	53	8	53	
T-stage							
T3	26	87	13	87	13	87	1.00
T4	4	13	2	13	2	13	
N-stage							
N0	11	37	9	60	2	13	0.02
N1	8	27	4	27	4	27	
N2	11	37	2	13	9	60	
TNM Stage							
II	11	37	9	60	2	13	0.01
III	19	63	6	40	13	87	
Adjuvant therapy							
No	19	63	12	80	7	47	0.06
Yes	11	37	3	20	8	53	

Table 2. All 58 endpoints analysed with reverse phase protein microarray analysis (RPMA) sorted by group.

58 endpoints analyzed by RPMA

Growth factors and receptors	
VEGFR2 (Y1175)	SD
PDGFR	TTS
PDGFRbeta (Y751)	
IGF I	
IGF I R (Y1135-36)/IR (Y1150-51)	TTS
TGFbeta	
NGF	
Wnt5a/b	TTS

Interleukins
IL6
IL10
ILI beta
IL8

EMT and EM remodelling	
Vimentin	
E-Cadherin	
Twist I	
MMP2	TTS
MMP9	
MMP14	
TIMP2	
TIMP3	

Protein Kinases	
Jak I (Y1022-23)	
Jak 2 (Y1007-8)	
Zap70 (Y319)/Syk (Y352)	SD
Akt (S473)	
Erk 1/2 (T202-Y204)	
FAK	
FAK (Y576-577)	
IRAK I	
p38MAPK (T180-Y182)	
Lck (Y505)	TTS

Downstreams	
SMAD 4	
SMAD 1/5/8	
Stat4 (Y693)	
Stat6 (Y641)	
Stat1 (Y701)	
Stat3 (Y705)	
Stat5 (Y694)	TTS
Beta Catenin (T41-S45)	
DKK I	
eNOS (S113)	SD
eNOS/NOSIII (S116)	

EM Composition
Collagen I

Fibroplastic component	
FSP/S100A4	TTS
alphaSMA	

Inflammatory component
CD45
CD5L
Arpc2

Transcription factors
NFkB.p65.S536
Egr I

Other markers	
CAV I	
CAV I (Y14)	
ICAM I	SD
cILAMINA	
SERPINA I	
Cox2	
OPN	
Podoplanin	
LDHA	

Legend:
SD = statistically different
TTS = a trend towards significance ($0.05 < p < 0.1$)

SD meaning statistically different between the stroma-high and stroma-low groups (p-value <0.05). TTS meaning a trend towards significance with a p-value between 0.05 and 0.1.

Data analysis

To explore the changes in the activation/phosphorylation and expression levels of different analytes mean comparison was used. A two-sample t-test was used for analytes that were normally distributed. For proteins/phosphoproteins presenting with asymmetric distribution the Wilcoxon rank sum test was performed. Data analysis was performed using SPSS version 19. All p values < 0.05 were considered statistically significant. To explore the interactions between proteins/phosphoproteins within the cellular compartments Spearman Rho correlation coefficients were calculated. Correlation maps were created with Gephi version 0.8.2. Only associations with a correlation coefficient ≥0.75 were included in the correlation maps.

RESULTS

The LUMC database consisted of 80 patients whose TSR could be scored and fresh frozen tissue was available. For this feasibility study, only cases with ≤30% stroma or ≥70% stroma were analysed. A total of 30 colon cancer samples (15 stroma-high and 15 stroma-low) were randomly selected from patients with histologically proven stage II and stage III colon cancer. The amount of stroma in the frozen tissue sample was double checked to be the same as the paraffin sample before including for analysis. Eleven patients were TNM stage II (37%) and 19 patients TNM stage III (63%). For detailed patient characteristics, see table 1.

Mean comparison analysis showed that 4 of the 58 analytes measured were statistically different between the two stroma groups. Phosphorylation of vascular endothelial growth factor receptor-2 (VEGFR-2) was significantly higher in the stroma-high group compared to the stroma-low group ($p=0.02$). Furthermore, zeta-chain-associated protein kinase 70 (ZAP70), endothelial nitric oxide synthase (eNOS) and intercellular adhesion molecule-1 (ICAM-1) were significantly lower in the stroma-high group compared to the stroma-low group ($p=0.01$, $p=0.04$ and $p=0.03$, respectively) (Figure 3).

Correlation analysis demonstrated more interconnections in the stroma-low group compared to the stroma-high group where proteins did not seem to trend together. When the analysis was limited to highly correlated pairs (≥0.75), some major clusters were identified (Figure 4). The stroma-low group showed to have two major nodes: eNOS and ARPC2 correlated pairs, respectively.

DISCUSSION

These results reveal the potential presence of biochemical derangements in the tumor stroma of tumors from patients with aggressive colon cancer with increased activation of VEGFR-2 and decreased activation of ZAP70, ICAM-1 and eNOS.

First, VEGFR-2 is one of the most prominent ligand-receptor complexes in the VEGF system. It can lead to endothelial cell proliferation, migration, survival and new vessel formation involved in angiogenesis [39]. High levels of VEGF expression are related to poorer survival and an increased rate of distant metastases in colorectal cancer patients [40]. To find altered levels of VEGFR in stroma-high patients correlates well

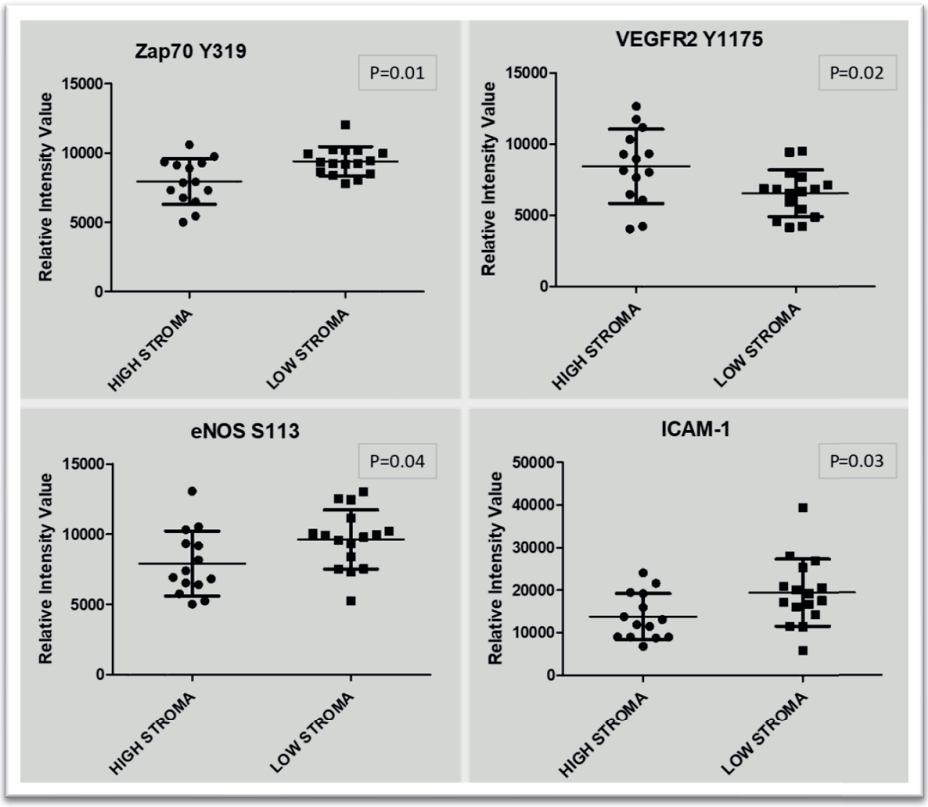


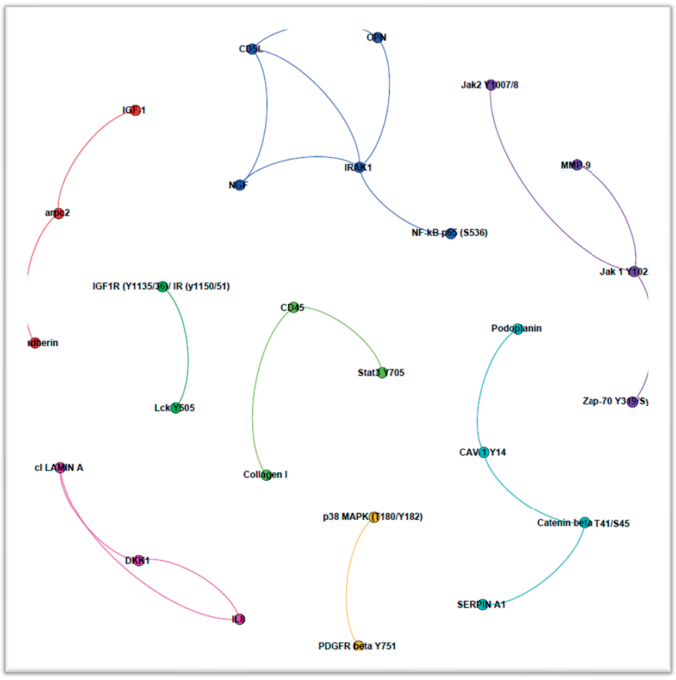
Figure 3. Significantly different analytes in stroma-high versus stroma-low group.

with the poorer survival of this group and could be an extra linking factor for new therapeutic strategies.

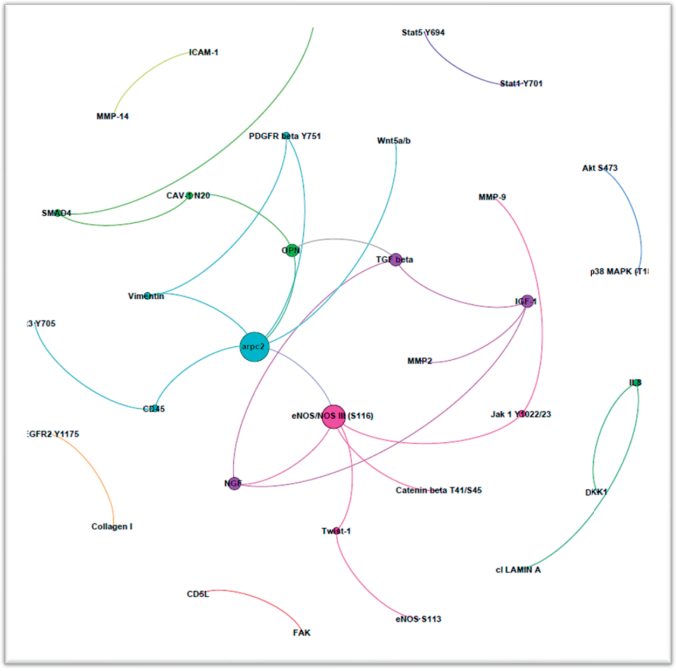
Considering the central role VEGFR plays in angiogenesis and cell migration, the results could suggest that anti-VEGFR targeted therapy could be considered for a pre-stratified group of patients with aggressive tumors with high recurrence rates. The Quick and Simple and Reliable trial (QUASAR2), is a phase III randomized trial of adjuvant capecitabine (CAP) ± bevacizumab (BEV) after complete surgical resection of high-risk stage II and stage III colorectal cancer [41]. Bevacizumab is a recombinant humanized monoclonal antibody that blocks angiogenesis by inhibiting vascular endothelial growth factor A (VEGF-A). VEGF-A is a growth factor protein that stimulates angiogenesis in a variety of diseases, especially in cancer. In this study, our group investigated whether this anti-angiogenic therapy might improve survival of patients with a stroma-high profile with a higher expression of VEGFR-2 and potentially increased angiogenesis. However, no benefit was found in response to treatment with

Figure 4. Correlation maps showing protein interactions and networks within the tumor stroma for a) for stroma-high and b) stroma-low samples. Only correlations with a coefficient ≥0.75 are shown in the maps.

A)



B)



bevacizumab when stratified for TSR [5]. Assessing different angiogenetic strategies and therapeutic options could have an additional value for improving survival of the stroma-high patient population.

Second, ZAP70 encodes an enzyme belonging to the protein tyrosine kinase family and plays a role in T-cell development and lymphocyte activation. It is used as a prognostic marker in identifying different forms of chronic lymphocytic leukaemia (CLL), where the expression of ZAP70 is associated with a significantly lower overall survival [42]. There is currently no available data on its role in CRC. In our study, we found a lower expression of ZAP70 in the stroma-high group. This correlates with our visual finding that stroma-high tumors have microscopically less lymphocytic infiltration compared to the stroma-low tumors. Further research is necessary to unravel the underlying mechanism and the possible clinical implications behind this.

Third ICAM-1 is a surface glycoprotein and is known to be a member of the immunoglobulin gene superfamily of adhesion molecules. It is expressed on vascular endothelium and plays a key role in the trans-endothelial migration of neutrophils and T-cell activation [43]. It has been suggested that ICAM-1 can inhibit cancer progression by activation of the host immune surveillance system by adherence to the extracellular matrix and thereby alleviating or eliminating metastasis of CRC [43, 44]. In our study, a lower expression of ICAM-1 was seen in the stroma-high population, correlating with a worse prognosis.

Lastly, eNOS is a gene expressed in the endothelium involved in the production of nitric oxide (NO), which plays a central role in maintaining endothelial cell functional integrity, regulating hemodynamics, and establishing collateral circulation [45]. Literature suggests that NO plays a key role in physiological regulations, including defence mechanisms against infectious disease and tumors [46]. A high level of expression of endothelial cell nitric oxide synthase (eNOS) in micro vessels in the tumor-adjacent area protects against tumor metastasis [47]. In our study, a higher expression was seen in the stromal tissue of the stroma-low group with better survival rates, correlating with this hypothesis.

While the four above discussed analytes are differently expressed among different groups, we also performed a correlation analysis (see correlation map in Figure 4). This correlation analysis showed that in addition to those detected differences, eNOS is a node that shows many interconnections in the tumor stroma within the stroma-low group. With the characteristics of eNOS as described above, it may be an important

analyte to contribute to the better prognosis of patients with a stroma-low tumor compared to the stroma-high group.

The other lead in the correlation map within the stroma-low group is ARPC2. Literature shows that ARPC2 in gastric cancers showed significant associations with large tumor size, lymph node invasion, and high tumor stage. In addition, in the same study, ARPC2-positive patients had lower recurrent free and overall free survival rates compared to ARPC2-negative patients [48]. Regarding breast cancer, ARPC2 is described to promote cancer proliferation and metastasis [49]. For colon cancer, literature so far only described an under-expression of ARPC2 in early colorectal cancer [50]. In our study, ARPC2 is equally expressed in the stroma-high and stroma-low group. However, ARPC2 shows many correlations and might be an important part of the stroma-low micro-environment network.

This pilot study showed the potential presence of biochemical derangements in the tumor stroma of tumors from patients with aggressive colon cancer with increased activation of VEGFR-2 and decreased activation of ZAP70, ICAM-1 and eNOS. Moreover, by focusing on the stroma-low group instead of the stroma-high group, there is a significantly higher expression of eNOS with many interconnections including ARPC2. These interconnections may play an important contribution to the prognosis of the stroma-low group. The preliminary findings in our study may offer a new lead for additional research to better understand the different tumor phenotypes of these two prognostically different groups based on their stroma amount.

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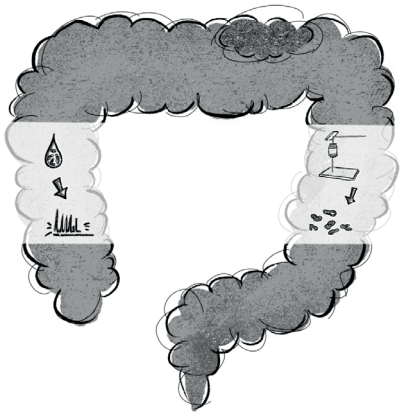

General Discussion and Future Perspectives

Colorectal cancer forms a major health burden. It is one of the most frequently occurring cancers worldwide next to lung and breast cancer [1]. In 2018, 14.200 newly diagnosed patients were reported in The Netherlands. Although the incidence rate of colorectal cancer (CRC) in the Dutch population has increased, the mortality rate has decreased due to continuous improvements in the diagnostic process and treatment options [2]. Nevertheless, CRC is still the second leading cause of cancer related deaths. Tumor stage is one of the most important prognosticators for colon cancer. Therefore, early diagnosis is of great importance to reduce disease-related mortality [3, 4].

This doctoral thesis analysed the pathologic and molecular characterizations of colorectal cancer, with a focus on the role of (diagnostic, prognostic and predictive) biomarkers and an aim to improve disease specific survival. The thesis was divided into two parts.

In the first part, the research analysed the role of proteomics as a *diagnostic* biomarker for early colorectal cancer detection. This part of the research is important because through its use as a diagnostic biomarker, proteomics may improve screening applications.

The second part of this research examined the role of stromatogenesis as a *prognostic* and *predictive* biomarker, and as such the role of stromatogenesis on risk stratification of colorectal cancer patients. This part is of great clinical relevance, because stromatogenesis in our research provides a robust, reliable biomarker. In addition, it gives future leads to develop new biomarkers that will contribute to risk stratification of colorectal cancer beyond clinical staging.

PART 1 PROTEOMICS AS A DIAGNOSTIC BIOMARKER

As mentioned before, early diagnosis of colon cancer is important to reduce disease-related mortality. Therefore, non-invasive screening methods can offer a vital improvement for survival. However, current screening protocols have a limited sensitivity and specificity [5-8]. We therefore chose to study whether the use of serum biomarkers to distinguish cancer patients from healthy persons could be a tool to improve screening programs. Serum is an ideal sample type for early detection markers since samples can be obtained in a straightforward, standardised manner at minimal cost, minimal risk and, most importantly, in a less-invasive manner compared to existing detection methods, such as colonoscopy [9].

Chapters 2 and 3 therefore focused on proteomic serum biomarkers. This could provide a non-invasive *diagnostic* biomarker. Mass spectrometry based proteomics (MS) is a technology used for mapping and identifying peptides and proteins in body fluids [10-14]. In **chapter 2**, an overview of protein profiling methods for CRC and breast cancer (BC) proteomic serum biomarkers is provided with translation to implementation in clinical setting and potential screening programs. Several case-control studies described favourable reports on serum protein profiling of BC and CRC. Comparing the reported sensitivities and specificities with current screening techniques, MS would appear to be a very promising tool. However, these results are likely to be overoptimistic when compared to a screening population. The described series analysed selected groups of patients with a priori a higher chance of having CRC compared to a screening population. As the control groups of those studies only consisted of healthy people, it is impossible to determine whether the discriminatory peaks are actually (colon) cancer specific or more general disease-specific. In addition, these studies used different sample processing methods. In order to apply MS in a routine clinical setting, collecting, measuring and processing of data must have strict protocols and guidelines to make it a robust and reproducible method [15, 16]. The current robotic platforms facilitate standardized methods and high throughput. It sometimes seems almost elusive to reproduce MS outcome into clinical practice, but focusing on analysing specific sets of identified proteins (targeted proteomics) instead of different protein spectra might give further direction into clinical translation. More comparative and prospective studies are needed to determine the value of MS in clinical practice and the possible superiority to other screening methods.

Therefore, we designed our study in the manner described in **chapter 3**: a case-controlled study that identifies proteomic profiles and their potential for colorectal cancer screening. For this study, a mass spectrometry based serum peptide and protein biomarker signature was found with a high discriminative power to distinguish CRC patients from healthy controls. The area under the curve (AUC) was 0.95 with a high specificity of 94-95%. Full automation of the preparation and analysis process in our robotic platform ensures standardization and robustness. The current screening with immunochemical faecal blood test (iFOBT) requires many additional colonoscopies. Almost 90% of those colonoscopies following a positive iFOBT are negative [17]. Colonoscopies are invasive procedures that are not without risks and often require sedation. It is time consuming and requires bowel preparation. Instead, the serum proteomics test is based on the analysis of one tube of peripheral blood. It is easy to apply, cheap and patient friendly with good sensitivity and specificity. Comparing this test performance in a population cohort and to the current screening methods may result in additional possibilities for less invasive screening programs. However, despite

the high discriminative power and automated handling, larger studies are essential to evaluate the 'tumor-specificity' of the obtained discriminative signatures.

Since conducting our research, a large number of additional CRC biomarkers were identified by proteomics using diverse approaches. Alnabulsi et al and Binetti et al, give a detailed review of recent achievements of clinical implementation of those biomarkers [18,19]. They again conclude that the clinical potential of proteomic biomarkers will not be fully determined without improvements in the validation process. Continued advancements in sample processing, detection technologies and computational analysis will gradually address the challenges in proteomics and hopefully enable the safe implementation in clinical setting.

PART 2 STROMATOGENESIS AS A PROGNOSTIC AND PREDICTIVE BIOMARKER

Chapters 4-6 focused on stromatogenesis and its possible role as a new *prognostic* and *predictive* biomarker. Stromatogenesis is the formation of new specific types of tumor stroma. Apart from the importance of early detection, the stage-independent outcome variability is a topic of great interest. Some patients with early stage CRC may show relapse, cancer progression and worse survival compared to other early stage CRC patients. To evaluate this risk stratification of colon cancer beyond current clinical staging, understanding the molecular heterogeneity enhances the ability to select patients in need of additional or adjusted treatment protocols [20]. Tumor stroma facilitates tumor cell invasion and migration. Therefore, tumor stroma and cancer cell interactions may be key elements in the puzzle of tumor survival, growth, invasion and metastasis [43=21]. The tumor stroma percentage in colon cancer patients has previously been reported by our research group as a strong independent prognostic parameter [22, 23]. Patients with a high stroma percentage within the primary tumor have a poor prognosis. In **chapter 4**, validation of the tumor-stroma ratio (TSR) as a prognostic biomarker in a large study population of the VICTOR trial is described, confirming the TSR to be an independent strong prognostic factor. Our study confirms that an increased amount of stromal involvement, even if it is detected in only a small part of the total tumor mass, can be linked to an unfavourable prognosis, independent of other prognostic parameters. Next to histopathological staging, the microsatellite instability (MSI) status is advised as an indicator for therapy choice and possible predictor for prognosis [24-27]. In this study the MSI status showed no significant difference in survival, but TSR and MSI were found to be associated. Furthermore, our high inter-observer agreement in this and previous studies indicates that the

TSR is a highly reproducible measurement. It is remarkable that a simple tissue-based parameter can possess such a high discriminative power without any additional costs. It would therefore be of great importance to implement TSR into daily routine diagnostics next to the TNM classification, to better predict prognostic outcome of CRC patients.

In our study, the worse prognosis of TSR high patients was again confirmed. However, there is no suitable therapy or even a lead for new therapy developments for this high-risk group. We therefore investigated the stromatogenesis process to discover perspectives for new treatment options. One of our hypotheses was that because one of the factors of tumor progression facilitated by the tumor stroma is angiogenesis, anti-angiogenetic therapy could help increase survival of this high-risk patient group. In **chapter 5**, we therefore evaluated the TSR in the QUASAR 2 trial. We investigated whether anti-angiogenic therapy might improve survival of patients with a stroma-high profile. The QUASAR 2 trial is a large phase III randomized trial of adjuvant capecitabine (CAP) ± bevacizumab after complete surgical resection of high-risk stage II and stage III colorectal cancer [28]. Bevacizumab is a monoclonal antibody against vascular endothelial growth factor, which therefore might interfere with the stromatogenesis. Importantly, although the study population only consisted of high-risk patients, the study confirmed again that TSR is an independent prognostic factor for colon cancer patients by showing that this parameter is strong enough to differentiate patients even in an already selected group. In addition, a worse survival for patients with vascular invasion was confirmed. Nonetheless, our hypothesis failed because no effect in disease free survival was seen with respect to additional bevacizumab treatment. Furthermore, a significant difference in survival was seen comparing groups with or without vascular invasion. And above that, a correlation between vascular invasion and stroma-high was seen, supporting the negative prognostic value of both high-risk factors. The relation between patients with a stroma-high tumor and vascular invasion has not been described earlier. This correlation could confirm the important role angiogenesis plays in the stromal environment.

But besides bevacizumab, different treatment regimens should be evaluated. Further knowledge of the stromal composition might lead to new targeted treatment regimens. In **chapter 6** we therefore evaluated this stromal composition to identify its activated pathways and the possible interactions for therapy targets. We described a pilot study where stromal tissue was analysed using laser capture microdissection coupled to broad-scale protein pathway activation mapping using reverse phase protein microarrays. We performed this pilot to try to better understand the way stromatogenesis originates and evolves and why patients with a stroma-high tumor

have a poor prognosis, what causes the aggressiveness of tumors with high stromal formation and what pathways are involved in this process. Patients with histologically proven stage II and stage III colon cancer were selected from the LUMC database. Reverse phase protein microarray was performed using microdissected tissue material to generate multiplexed pathway profiling.

Statistical comparison showed the potential presence of biochemical derangements in the tumor stroma from patients with stroma-high colon cancer with increased activation of VEGFR-2 and decreased activation of ZAP70, eNOS and ICAM-1 compared to stroma-low tumors. VEGFR2 is one of the most prominent ligand-receptor complexes in the VEGF system. It can lead to endothelial cell proliferation, migration, survival and new vessel formation involved in angiogenesis [29]. High levels of VEGF expression are related to poorer survival and an increased rate of distant metastases in colorectal cancer patients [30]. ZAP70 encodes an enzyme belonging to the protein tyrosine kinase family and plays a role in T-cell development and lymphocyte activation. It is used as a prognostic marker in identifying different forms of chronic lymphocytic leukaemia (CLL). The expression of ZAP70 is associated with a significantly lower overall survival [31]. Its role in CRC is not described yet. Our study showed a lower expression of ZAP70 in the stroma-high group. This correlates with our visual finding of stroma-high tumors having microscopically less lymphocytic infiltration compared to the stroma-high tumors. Further research is necessary to unravel the mechanism and the possible clinical implications behind this. eNOS is known to be involved in the production of nitrogen oxide (NO) through L-arginine. Literature suggests that NO plays a key role in physiological regulations, including defence mechanisms against infectious disease and tumors [32]. ICAM-1 is a surface glycoprotein and is known to be a member of the immunoglobulin gene superfamily of adhesion molecules. It is expressed on vascular endothelium and plays a key role in the trans endothelial migration of neutrophils and T-cell activation [33]. It has been suggested that ICAM-1 can inhibit cancer progression by activation of the host immune surveillance system by adherence to the extracellular matrix and thereby alleviating or eliminating metastasis of CRC [33, 34].

Correlation analysis also showed more interconnections in the stroma-low group compared to the stroma-high group. The stroma-low group showed two major interconnection nodes: eNOS and ARPC2. Furthermore, within the stroma-low group, there is a significantly higher expression of eNOS with many interconnections including ARPC2. With the characteristics of eNOS as described above, it may be an important player contributing to the better prognosis of patients with a stroma-low tumor compared to the stroma-high group.

The other lead in the correlation map within the stroma-low group is ARPC2. In literature ARPC2 in gastric cancers showed significant associations with large tumor size, lymph node invasion, and high tumor stage. In addition, in the same study ARPC2-positive patients had lower recurrent free and overall free survival rates compared to ARPC2-negative patients [35]. In breast cancer, ARPC2 is described to promote cancer proliferation and metastasis [36]. In colon cancer, so far only an under expression of ARPC2 in early colorectal cancer is described [37]. In our study, ARPC2 is equally expressed in the stroma-high and stroma-low group. But ARPC2 shows many correlations and might be an important part of the stroma-low micro-environment network.

The aforementioned interconnections might play an important contribution to the favourable prognosis of the stroma-low group. These findings in our study could give a new lead for additional research to better understand the different tumor phenotypes of these two prognostically different groups based on their stroma amount.

FUTURE PERSPECTIVES

Proteomics future prospects

The field of proteomics is constantly changing. In earlier days biomarker discovery was performed using protein profiling or (untargeted) proteomics. Nowadays targeted quantitative proteomics, with predefined set of biomarkers is performed. Quantitative proteomics using mass spectrometry allows for system-wide identification and quantification of proteins and targeted proteomics applications. Quantitative mass spectrometry analyses can detect and quantify thousands of proteins in a single experiment. Furthermore, combining laser capture microdissection and proteomics techniques is a promising way to find significant differentially expressed proteins in target tissues [38, 39]. Furthermore, like mentioned earlier, the challenge of clinical implementation depends largely on the possibility of a reproducible and well validated biomarker. Continued advancements in knowledge, technologies and computational analysis will hopefully enable the safe implementation of proteomic biomarkers in clinical setting.

TSR Prospective multicentre study

To further refine the prognostic prediction strategies of CRC patients, it would be of great importance to implement TSR into daily routine diagnostics next to the TNM classification. It is a low-cost test, performed on standard HE slides and requiring only a small amount of time. The TNM Evaluation Committee (UICC) and the College of

American Pathologists (CAP) stated the TSR has the potential to be included in the TNM staging algorithm but needs validation in a prospective cohort. Therefore, the UNITED study has been designed [40]. This international multicentre study investigates the reproducibility of scoring the TSR amongst pathologists, using an E-learning module. Stage II and III colon cancer patients are simultaneously included to validate the prognostic value of the TSR in a European prospective observational cohort. The inclusion of patients is still ongoing. After the results of this prospective study are published, which confirm that the TSR is an independent strong diagnostic biomarker, we expect the TSR to be implemented next to the routinely used TNM classification.

Stromatogenesis

The mechanism by which tumor stroma facilitates tumor progression has not yet been fully unravelled. However, a key hypothesis is that stroma producing factors influence local and systemic inflammation, tumor pH and tumor metabolism [41]. An improved understanding of tumor and stroma metabolism could give insights and possible leads for new therapy strategies. Normal differentiated cells primarily rely on mitochondrial oxidative phosphorylation to generate the energy needed for cellular processes. In contrast, most cancer cells rely on aerobic glycolysis, a phenomenon called "the Warburg effect" [42]. Aerobic glycolysis is an inefficient way to generate energy. The advantage it might confer to cancer cells has been unclear, but this process might be facilitated by the tumor-supporting stroma. Giatromanolaki et al. reported that increased tumor cell expression of enzyme pathways associated with anaerobic metabolism and lactate extrusion, including lactate dehydrogenase isoenzyme 5 (LDH-5) and monocarboxylate transporter 1 (MCT-1), increased the ability of cancer-associated fibroblasts to uptake and oxidate lactate, supporting tumor cell metabolism [21].

As Roseweir et al. described in their study that the combination of TSR and tumor cell expression of cytoplasmic MCT-2 or nuclear LDH-5 is associated with poor prognosis for stage I-III CRC. Moreover, the combination of TSR and nuclear LDH-5 was significantly associated with increased tumor budding and decreased stromal CD3+ T-lymphocytes. Tumor budding is associated with poor prognosis in CRC and is thought to be the histological representation of epithelial-mesenchymal transition. Decreased T-lymphocytes might suggest that highly metabolically active tumor cells utilize metabolites that are needed by T-lymphocytes to survive and function [43,44]. This supports the hypothesis that one mechanism by which increased stromal invasion promotes tumor progression is through modulation of tumor metabolism. Blocking this metabolic support could be of great therapeutic relevance. Inhibitors of lactate dehydrogenase or blockers of monocarboxylate transporters would severely compro-

mise the metabolic activity and may provide promising therapeutic targets for patients with stroma-high CRC [21,44].

Biopsy TSR

The TSR is assessed on resection specimen of CRC, but it could be interesting to evaluate the value of TSR pre-operatively on biopsy tissue. To that end, it seems feasible to examine the tumor microenvironment on endoscopic biopsy specimen. Park et al. analysed biopsies and resection specimens and found stroma-high in biopsies predicted stroma-high in resected specimens associated with cancer specific survival [45]. However, due to intratumor heterogeneity this also has its limitations. A single biopsy might not adequately represent the stromal makeup of the tumor. In addition to tissue biopsies, liquid biopsies are described as a new method for early detection and tracking of biomarkers during treatment, especially in blood [46]. Zheng et al. suggested that some of the essential interactions between proliferating cells and tumor stroma can in part be monitored through stromal liquid biopsies where the extracellular proteins are found as a proteomic pattern in the general blood circulation (serum) of patients with different types of cancer [47,48]. More research in this area therefore looks promising for early prediction of prognosis or even prediction and monitoring therapeutic benefit in both stroma-high and -low CRC patients.

Digitalizing, Artificial Intelligence and Deep Learning

Recent years, pathology has moved towards a more digitalized workflow. Pathology sections are more and more scanned for digital viewing on a computer instead of examined by the pathologist using conventional microscopy. In this shift towards a digital workflow, automation of tissue parameters and even deep learning to evaluate specimen is of growing interest. Current research is exploring possibilities of developing new algorithms to support the pathologists in daily practice and to reduce their workload. Zhao et al. confirm again the prognostic effect of the TSR for overall survival of colon cancer patients, showing the robustness of the TSR method. But above all they show the possibility to quantify the tumor-stroma percentage by artificial intelligence. Although there are still challenges to overcome, this is a huge step forward. One of those challenges is, for example, the importance of stain normalization before running the algorithm, because of its sensitivity to variation in colours [49]. Skrede et al. recently published an article in the Lancet describing the use of a prognostic marker algorithm based on TSR, which was developed by using deep learning methods [50]. While artificial intelligence may play a role in future clinical decision making, caution has to be taken. For example, Specogna et al. stated important limitations within the training set of Skrede et al. and also an automation bias. A system that is automated is usually entirely data driven and not trained to understand why. Using a causal perspec-

tive, an outcome occurs. Attention should be given to how learned biases might relate to errors eventually translated into clinical decisions with the potential to harm patients. Artificial intelligence, automation and deep learning can bring research to a next level. However, they are unlikely to eliminate the need for expert human intervention, even though they could allow for greater efficiency. Prospective validation studies are needed to assure the safeness of implementation for routine clinical use [51].

Introduction of new biomarkers in the clinic

Implementation of new biomarkers in clinical guidelines and daily practice is time consuming and may take more than a decade. Clinical guidelines should be based on the highest quality of evidence leading to the best available treatment and a standardized approach to patient care [52]. New robust biomarker application is challenging sometimes because of methodological aspects, such as robustness and reproducibility, related to the quality of the technology, the sample, or sometimes just because of the complexity of the tumor biology. More efficient sampling and the use of high-sensitive methodologies within clinical multidisciplinary trials that meet the highest quality standards may overcome the influence of tumor heterogeneity and result in reproducible highly reliable biomarkers. But even when biomarkers fulfil all the criteria, implementation might eventually not be achieved [53].

CONCLUSIONS

This thesis highlights, firstly, the importance of early CRC detection by presenting results of a CRC *diagnostic* proteomic biomarker signature with high discriminative power. Secondly, the strong robust, independent *prognostic* TSR biomarker confirms to be of important clinical value. The TSR has the ability to stratify colon cancer patients according to their prognostic outcome in a highly reproducible and low-cost manner. It has shown to link patients with a high intra tumor stromal content and a worse prognosis. Literature shows a wealth of evidence that supports this prognostic value in CRC as well as in other cancers. This PhD research therefore concludes that it should be implemented in the official guidelines of the TNM classification to improve stratification for CRC patients in daily routine pathological evaluation. The prospective, international, multicentre UNITED study will hopefully overcome the last hurdle for this clinical implementation. Lastly, this thesis offers more insight in the elusiveness of the tumor microenvironment and stromatogenesis that contributes to the aggressiveness of some CRC tumors. The biological differences, interconnections and changes in the microenvironment presented give multiple leads for further research and new personalized treatment possibilities.

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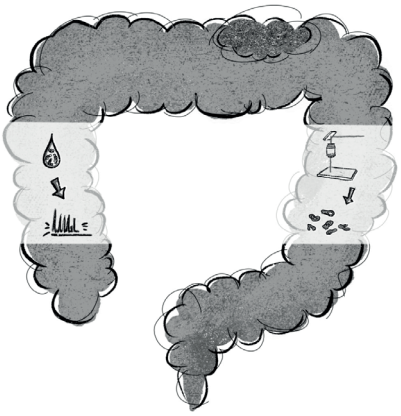

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Nederlandse samenvatting
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NEDERLANDSE SAMENVATTING

Darmkanker is een van de meest voorkomende vormen van kanker. Bij vrouwen staat het na borstkanker op de tweede plaats en bij mannen na long en prostaatkanker op de derde plaats. Bovendien is het de derde en vierde belangrijkste oorzaak van kanker gerelateerde sterfgevallen bij respectievelijk vrouwen en mannen [1].

Vroege opsporing is cruciaal. Darmkanker ontstaat door een langzame ontwikkeling van normaal darmslijmvlies naar afwijkend slijmvlies, vaak begint het bijvoorbeeld met een poliep die uit kan groeien naar darmkanker. Het vermoedelijke verloop van darmkanker (de prognose) hangt voornamelijk af van hoe ver de kanker is gevorderd: het zogenoemde tumorstadium. Daarom is het vroege opsporen van groot belang om de overlevingskansen van mensen met darmkanker te verbeteren [2, 3].

De kans op het overleven van darmkanker is de laatste jaren toegenomen, enerzijds door het verbeteren van de vroege opsporing met behulp van het bevolkingsonderzoek en anderzijds door nieuwe en verbeterde behandelingen.

Het inschatten van het stadium van de tumor gebeurt op dit moment met behulp van het TNM stadium. T staat voor tumor, de mate van doorgroei van de tumor door de darmwand, N voor node = lymfklieren waar wel of geen uitzaaiingen in zitten en M voor metastase, eventuele uitzaaiingen in andere organen. Ondanks deze stadiëring zien we dat er grote verschillen te zien zijn in de overleving en ook terugkeer van ziekte bij patiënten die eigenlijk in dezelfde risico groep zitten. Dit suggereert dat er nog andere verschillen per tumor zijn waar we verder naar moeten kijken, zodat er een nog betere inschatting van de eigenschappen van de tumor per patiënt gemaakt kan worden [4]. Met als uiteindelijk doel de behandeling per patiënt zo af te stemmen op de specifieke tumor eigenschappen dat de uitkomsten nog beter worden.

In dit proefschrift analyseren we deze karakteriseringen van darmkanker. We richten ons daarbij op de rol van biomarkers. Een biomarker, ook wel biologische marker genoemd, is een meetbare indicator die zowel normale biologische processen, alsook bijvoorbeeld processen tijdens een ziekte kan weergeven [5].

Biomarkers worden vaak gedefinieerd in overeenstemming met hoe ze worden toegepast. Dit onderzoek richt zich op drie specifieke subtypes van biomarkers: diagnostische, prognostische en predictieve biomarkers en hun rol bij het verbeteren van de uitkomst van darmkanker.

Deel 1 Proteomics als diagnostische biomarker

In het eerste deel onderzoeken we de rol van proteomics als diagnostische biomarker voor vroege detectie van darmkanker. Proteomics is de studie van het proteoom: alle eiwitten (proteïnen) van een organisme of een deel van een organisme. Proteomics heeft als doel om zowel kwantitatief als kwalitatief alle functionele eiwitten van een organisme in kaart te brengen. Het is gebaseerd op het scheiden en nauwkeurig in kaart brengen van complexe eiwitmengsels. Zo kunnen bijvoorbeeld eiwitten met een veranderd expressiepatroon in bloed worden opgespoord en in verband worden gebracht met de aanwezigheid van een tumor.

Het gebruik van biomarkers in bloed om kankerpatiënten te onderscheiden van gezonde personen kan een hulpmiddel zijn om screeningsprogramma's te verbeteren. Bloed of serum (=bloedvloeistof waaruit rode en witte bloedcellen en bloedplaatjes zijn verwijderd) is een ideaal monstertype voor markers voor vroege opsporing, omdat het op een eenvoudige, gestandaardiseerde manier kan worden verkregen zonder hoge kosten of grote gezondheidsrisico's voor de patiënt. [6]

In **hoofdstuk 2** wordt een literatuuroverzicht gegeven van proteomics onderzoeken die het potentieel hebben om te worden geïmplementeerd in de kliniek en nationale screeningprogramma's voor darmkanker en borstkanker. In **hoofdstuk 3** hebben we een eiwit profiel in het serum geïdentificeerd met een hoog onderscheidend vermogen tussen darmkanker en gezond. Dit is geautomatiseerd geanalyseerd op een robot platform zodat het steeds exact op dezelfde manier gebeurd en er grote hoeveelheden samples tegelijk geanalyseerd kunnen worden. Het is een relatief eenvoudige en goedkope test die mogelijkheid heeft om de huidige darmkanker screening te verbeteren. Ondanks het hoge onderscheidende vermogen van dit profiel en de geautomatiseerde verwerking, zijn grotere studies echter essentieel om de 'tumorspecificiteit' van de verkregen discriminerende handtekeningen te evalueren. Met andere woorden of deze handtekening echt specifiek bij darmkanker past en niet een weerspiegeling is van bijvoorbeeld een meer algemeen ziekte proces. Dit blijft een grote uitdaging, echter voortdurende vooruitgang in bloedverwerking, meetmethoden en computer analyses zullen hopelijk de betrouwbare implementatie in een in de kliniek mogelijk maken.

Deel 2 Stromatogenese als een prognostische en voorspellende biomarker

In **hoofdstukken 4-6** onderzoeken we de rol van stromatogenese en de rol van de tumor-stroma ratio (TSR) als een prognostische en voorspellende biomarker. Stromatogenese is de vorming van (tumor) stroma, een soort bindweefsel. Er zijn veel soorten stroma vorming, zoals bijvoorbeeld littekenvorming bij de genezing van een

wond. Het type stroma waar dit onderzoek over gaat, bij kwaadaardige tumoren, is een heel ander soort weefsel.

De afgelopen jaren is duidelijk geworden dat de omgeving waarin tumorcellen zich bevinden, het tumor-stroma dat met name bestaat uit bindweefsel (fibroblasten), bloedvaten en immuuncellen, belangrijk is voor de tumor groei en de ontwikkeling van uitzaaiingen. Daarom kunnen tumor-stroma en kankercel-interacties sleutelelementen zijn in de puzzel van tumoroverleving, groei, invasie en uitzaaiing [7]. Dit geeft aanknopingspunten voor eventuele nieuwe, beter op de patiënt afgestemde behandelmethoden.

Een prognostische biomarker die wij verder onderzoeken is de tumor-stroma ratio (TSR). Deze marker kan worden beoordeeld door eenvoudige microscopische analyse van weefselcoupes (stukjes van de tumor die na een operatie worden onderzocht). Dit weefsel wordt nu ook al standaard door de patholoog onder de microscoop bekeken. Het is dus één extra score naast de al standaard te scoren parameters. Deze analyse is snel, goedkoop en betrouwbaar. Eerder onderzoek door onze groep toonde aan dat de TSR bij patiënten met darmkanker een sterke onafhankelijke prognostische voorspeller is [8,9]. Patiënten met een hoog stromapercentage binnen de primaire tumor hebben een slechter ziektebeloop, ook wel een slechte prognose genoemd.

In **hoofdstuk 4** wordt de bevestiging (ook wel validatie genoemd) van de TSR als een prognostische biomarker in een grote studiepopulatie met 710 patiënten uit de VICTOR trial beschreven. Deze studie bevestigt dat de TSR een onafhankelijke sterke prognostische factor is. [10, 11]. Als er veel stroma weefsel aanwezig is in het meest invasieve deel van de tumor, dan is dit geassocieerd met een ongunstige prognose, onafhankelijk van andere prognostische parameters. Het meest invasieve deel van de tumor is de plek waar de tumor het diepst in de darmwand groeit of door de darmwand heen groeit. Zoals hierboven ook benoemd is het een eenvoudig te bepalen score naast de al te scoren parameters. Het is opmerkelijk dat een eenvoudige op weefsel gebaseerde parameter zo'n groot onderscheidend vermogen kan hebben zonder extra kosten. Het zou daarom van groot belang zijn om TSR naast de huidige classificatie in de dagelijkse routinediagnostiek te implementeren, om de prognostische uitkomst van darmkanker patiënten beter te voorspellen.

Hiermee is de slechtere prognose van patiënten met een hoge TSR opnieuw bevestigd. Er is echter nog geen geschikte therapie of zelfs geen aanknopingspunt voor nieuwe therapieontwikkelingen voor deze risicogroep. We hebben daarom het ontstaan van

stromaweefsel, het stromatogenese proces onderzocht om aanknopingspunten voor nieuwe behandelingsopties te ontdekken.

We weten dat tumor-stroma veel bloedvatvorming heeft en dat bloedvatvorming ook een mechanisme is dat de tumor kan helpen zich uit te breiden [12,13]. Hierdoor was een van onze gedachten dat medicatie specifiek tegen bloedvatvorming (angiogenese) misschien goed zou werken voor deze patiëntengroep.

In **hoofdstuk 5** hebben we daarom de TSR in de QUASAR 2-studie geëvalueerd en het effect van anti-angiogenese medicatie (bevacizumab)[14].

Onze hypothese dat anti-angiogenese medicatie een betere ziekte vrije overleving zou opleveren binnen de TSR hoog groep kon helaas niet worden bevestigd. Ofwel onze groep patiënten met veel tumor-stroma in hun tumor hadden geen verbetering van hun prognose na behandeling met bevacizumab.

Wel werd ook in deze studie opnieuw bevestigd dat de TSR een onafhankelijke prognostische factor is voor darmkankerpatiënten. Daarnaast werd een significant slechtere overleving voor patiënten met vasculaire invasie (aanwezigheid van tumorcellen in de bloedvaten van de tumor) bevestigd.

Ook werd er een verband gezien tussen vasculaire invasie en de stroma-hoog groep, wat de voorspellende waarde van beide hoog risicofactoren ondersteunt. De relatie tussen patiënten met een stroma-hoge tumor en vasculaire invasie is niet eerder beschreven. Deze correlatie zou de belangrijke rol die angiogenese speelt in de stromale omgeving kunnen bevestigen en kan mogelijk toch een aanwijzing zijn voor het belang van een andere anti-angiogenese therapie dan bevacizumab bij deze patiëntengroep.

Evaluatie van de moleculaire architectuur van het tumor-geassocieerde stroma bij patiënten met darmkanker

Om de overlevingsverbetering van darmkankerpatiënten effectief aan te pakken, is het van groot belang om te begrijpen waarom patiënten met een hoog stromapercentage een slechte prognose hebben, wat de agressiviteit van veel stroma vorming veroorzaakt en welke stappen bij dit proces betrokken zijn. Om de architectuur van het tumor-geassocieerde stromaweefsel te evalueren, wordt in **hoofdstuk 6** een studie beschreven waarbij we met een specifieke laser techniek (laser capture microdissectie) de stroma cellen heel gedetailleerd selecteren uit het tumor weefsel. Hierbij worden zowel bij stroma laag als stroma hoog weefsel monsters alle stroma cellen geselecteerd en geïsoleerd en met een specifieke eiwit analyse (reverse phase protein

microarray) verder bekeken [15,16,17,18]. Om verschillen te kunnen ontdekken in de samenstelling van het stroma in beide groepen werd gezocht naar de aan- of afwezigheid van 58 specifieke eiwitten.

Statistische vergelijking toonde een verschil in de aanwezigheid van verschillende eiwitten (VEGFR-2, ZAP70, eNOS en ICAM-1). Deze stoffen kunnen als je gedetailleerd naar hun afzonderlijke werking kijkt bijdragen aan de betere prognose van patiënten met een stroma-lage tumor in vergelijking met stroma-hoog patiënten.

Verdere analyse liet hiernaast nog onderlinge verbindingen in de verschillende groepen zien (tussen eNOS en ARPC2 in de stroma laag groep). Deze bevindingen zouden een nieuwe aanzet kunnen geven voor aanvullend onderzoek om de verschillende tumortypes van deze twee prognostisch verschillende groepen beter te begrijpen op basis van hun stroma-hoeveelheid. En zo mogelijk hierop ook behandelopties te kunnen aanpassen.

TOEKOMSTPERSPECTIEVEN

Het voornaamste doel is om de gevonden resultaten te implementeren in de dagelijkse klinische praktijk naast de reeds bestaande methoden. Om hoog risico patiënten te selecteren die aanvullende of aangepaste therapie nodig hebben of juist laag risico patiënten die misschien juist geen aanvullende therapie (meer) nodig hebben.

Om dit voor de TSR te bereiken is een grote internationale studie opgezet (de UNITED-studie) [19]. Als de resultaten van deze studie bekend zijn en opnieuw bevestigen dat het een waardevolle prognostische marker, dan voldoet de TSR aan alle voorwaarden om veilig in de dagelijkse praktijk toe te passen.

Naast het onderzoeken van tumor weefsel na een operatie zou het ook interessant kunnen zijn om de waarde van het tumor-stroma al voor de operatie op biopsieweefsel te evalueren. Bijvoorbeeld bij biopten verkregen bij een inwendig darmonderzoek (colonoscopie) dat nu onderdeel is van de huidige bevolkingsscreening voor darmkanker. Een aantal onderzoeken beschrijven veelbelovende resultaten maar ook uitdagingen, bijvoorbeeld dat één biopt mogelijk niet de hele aard van de tumor goed weer kan geven. Meer onderzoek op dit gebied is nodig maar het kan veelbelovend zijn voor vroege voorspelling van de prognose of zelfs voorspelling en monitoring van therapeutisch voordeel bij patiënten met zowel een stroma-hoge als stroma-lage darm tumor.

Hiernaast is het digitaliseren van analyses ook een vernieuwing van de afgelopen jaren. Weefsel samples worden steeds vaker gescand en digitaal geanalyseerd in plaats van door de patholoog met een conventionele microscoop. Dit is een enorme stap voorwaarts, maar heeft nog veel uitdagingen. Kunstmatige intelligentie kan mogelijk in de toekomst een rol spelen bij analyse en klinische besluitvorming, maar met grote voorzichtigheid. Een geautomatiseerd systeem is meestal volledig data gedreven en niet getraind om te begrijpen waarom. Meer studies zijn nodig om de veiligheid van implementatie hiervan voor routinematig klinisch gebruik te verzekeren [20].

CONCLUSIE

In dit proefschrift beschrijven we een eiwitprofiel in bloed waarmee we darmkanker patiënten en gezonde personen kunnen onderscheiden. Mogelijk is dit een manier om darmkanker op een minder invasieve manier vroeg op te sporen. Als we een betere selectie kunnen maken welke patiënten een verhoogd risico hebben op darmkanker en daarvoor een colonoscopie (inwendig darmonderzoek) moeten ondergaan dan kunnen mogelijk een aantal onnodige colonoscopiëen achterwege worden gelaten.

Daarnaast laten we zien dat de stroma marker (TSR) die wij in meerdere onderzoeken beschrijven een goed inzicht geeft over de prognose van een patiënt met darmkanker. Te verwachten is dat deze stroma marker na de bevestiging in de UNITED studie toegevoegd zal worden aan de standaard scoring van darmkanker patiënten. Hopelijk gaat deze stroma marker ons in de nabije toekomst dan ook helpen een betere risico inschatting te maken en daardoor ook betere onderbouwde patiënt gerichte keuzes te maken voor het geven van wel of geen aanvullende behandeling zoals chemotherapie na een operatie. Denk bijvoorbeeld aan het weglaten van chemotherapie voor een oudere kwetsbare patiënt met een stroma-laag profiel.

Tenslotte geven de onderzoeken in dit proefschrift meer inzicht in de omgeving van de tumorcellen. De belangrijke rol die deze cellen spelen in de interactie met de tumor cellen. Hierdoor hebben we nieuwe aanknopingspunten om te begrijpen waarom sommige darmtumoren agressiever zijn dan andere. Aanknopingspunten die kunnen leiden tot verder onderzoek naar nieuwe behandelmogelijkheden afgestemd op de specifieke tumor eigenschappen per patiënt. Met als doel een beter ziektebeloop en een betere overleving na te streven voor patiënten met darmkanker.

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CURRICULUM VITAE

Anouck Huijbers was born on 11 October 1982 in Alphen aan den Rijn, The Netherlands. In 2008, she obtained her medical degree in Leiden. She then worked as a surgical resident (not in training) in the HagaZiekenhuis The Hague for 1.5 years with the objective to become a surgeon.

She then started her PhD project at the Surgical Oncology research group of Leiden University Medical Centers (LUMC) department of surgery, supervised by Prof. Dr.R.A.E.M. Tollenaar and Dr.W.E.Mesker. During the first 2.5 years, Anouck was a fulltime PhD researcher of which she spent 4 months at the National Cancer Institute C.R.O., Aviano, Italy under supervision of Dr.C.Belluco and 6 months at the George Mason University, Washington D.C., USA under the supervision of Prof. Dr. L.A. Liotta and Prof. Dr. E.F. Petricoin. In Italy and the USA she performed additional analysis to investigate the tumor stroma molecular architecture and mechanisms behind it.

In 2012, she was admitted to the Dutch surgical training program at LUMC. During this six years of surgical training Anouck worked at The Hague Medical Center (2012-2016), LUMC (general surgery (2016) and cardiothoracic surgery (2017)), Alrijne Hospital (2016) and Antoni v Leeuwenhoek (National Cancer institute (2018)).

In 2018, she finished her surgical training as a specialist in thoracic, gastro-intestinal and oncological surgery.

After a trip around the world with her husband, she came back to pursue her career as a lung surgical fellow at The Hague Medical Center in March 2019. Since September 2019, she holds a permanent position as a thoracic, gastro-intestinal and oncological surgeon at Dijklander Hospital, Hoorn, The Netherlands.

DANKWOORD

Mijn proefschrift is af! Gedurende het gehele traject zijn er velen geweest die mij op welke manier dan ook hebben gesteund en geholpen om tot dit eindresultaat te komen. Ik ben iedereen die hierbij betrokken is geweest dan ook heel erg dankbaar. Zonder iemand te kort te doen wil ik hieronder nog een aantal mensen persoonlijk bedanken.

Mijn promotor prof. dr. R.A.E.M. Tollenaar. Beste Rob, bedankt dat je me deze mogelijkheid hebt gegeven. Ook als we elkaar lang niet hadden gesproken wist jij met een half woord direct weer de essentie aan te stippen en de kritische vragen te stellen die nodig waren mij weer op het juiste spoor te krijgen. Nog steeds begrijp ik niet hoe je dat altijd weer voor elkaar krijgt. Dank voor al je hulp en vertrouwen.

Mijn co-promotor Dr. W.E. Mesker. Lieve Wilma, zonder jou zou dit proefschrift niet bestaan. Hoe vaak je me wel niet hebt geholpen de afgelopen jaren om door te gaan. De jaren dat ik in het LUMC werkte heb ik heel veel aan onze periodieke fijne overleg momenten gehad. Naast de noodzaak om wetenschappelijke progressie te maken verloor jij nooit het persoonlijke aspect uit het oog. De uitwisseling met Italië en de USA die voor mij heel plotseling en niet op een beter moment konden komen, toverde jij even uit je mouw. Nadat ik uit het LUMC weg was hadden we regelmatig contact en zorgde jij ervoor dat ik ondanks mijn klinische werkzaamheden mijn proefschrift niet uit het oog verloor. Dank voor al je geduld, je vertrouwen en fijne samenwerking.

Gabi, jouw dosis doorzettingsvermogen heeft mij enorm geholpen. Van meedenken bij zijsporen, lab ondersteuning, uren achter de microscoop en zelfs een werkbezoek in de USA, dank je voor al je hulp en gezelligheid.

Dear colleagues from the George Mason University in the USA and from CRO in Italy. Thank you for collaborating with me on this thesis and for all the good times between lab technical hours.

Cee Leven, het is al een hele tijd geleden dat we met elkaar op afdeling C11 de mooiste dagen hadden. Zonder die dagen was mijn research tijd niet hetzelfde geweest. Inhoudelijk sparren om tot de mooiste nieuwe inzichten te komen, in de vroege ochtend van den haag naar leiden racefietsen tussen de konijnen door, deadlines, last minute presentaties maken, mantelpakjes op de kamer voor noodsituaties, congres avonturen en veel persoonlijke verdieping. Work hard, play hard. Bedankt allemaal voor deze fantastische tijd.

Marieke, bedankt voor jouw fijne kritische blik en finale reviews. Chantal voor de creatieve ondersteuning en Do en Saar voor de vele wetenschappelijke discussies na werktijd.

En natuurlijk mijn familie, in het bijzonder mijn ouders en zus, dank voor al jullie steun, liefde en motivatie.

Lieve Dirk, heel erg bedankt voor al jouw hulp, geduld en vertrouwen. Nooit was het een probleem als ik naast mijn volle werkschema ook nog tijd nodig had voor mijn proefschrift. Zelfs tijdens onze reizen motiveerde jij me om verder te gaan. Je zocht het terrasje met het mooiste uitzicht om mijn laptop open te klappen en het schrijven zo leuk mogelijk te maken. Dank je dat je er altijd voor me bent.