Prognostic and predictive biomarkers in colorectal cancer. Towards precision medicine

Reimers, M.S.

PROGNOSTIC AND PREDICTIVE BIOMARKERS IN COLORECTAL CANCER

Towards precision medicine

Marlies Suzanne Reimers

Prognostic and predictive biomarkers in colorectal cancer

Towards precision medicine

Marlies Suzanne Reimers

Prognostic and predictive biomarkers in colorectal cancer

Towards precision medicine

Proefschrift

Ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus prof. mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 8 januari 2015
klokke 15.00 uur

door

Marlies Suzanne Reimers
geboren te Dordrecht
in 1984

PROMOTIECOMMISSIE:

Promotor	Prof. dr. C.J.H. van de Velde
Co-promotor	Dr. P.J.K. Kuppen Dr. G.J. Liefers
Overige leden	Prof. J. Morreau Prof. dr. A.J. Gelderblom Prof. dr. R. Fodde

ISBN: 978-94-6169-591-8

Layout and printing: Optima Grafische Communicatie, Rotterdam, The Netherlands

This thesis is also available as an e-pub. *www.e-pubs.nl?epub=m.reimers*

This thesis was supported by an unrestricted grant from the Sloos-Alandt family. Printing of this thesis was financially supported by: Amgen B.V., Bayer B.V., Boehringer Ingelheim B.V., Pfizer B.V., Roche Nederland B.V., Sanofi-Aventis Netherlands B.V., Sysmex Nederland B.V. en Chipsoft.

CONTENTS

CHAPTER 1

General introduction

INCIDENCE AND AETIOLOGY

Colorectal cancer (CRC) is the third most common cancer and is one of the major contributors to cancer-related deaths worldwide [1;2]. Approximately 20-25% of patients with CRC already have metastatic disease at the time of diagnosis and 20-25% of patients will develop metastases during disease progression as well, resulting in a 40-45% high mortality rate [3;4]. CRC can be divided in colon cancer, where the development of tumors ranges from the caecum to the sigmoid, and rectal cancer, that ranges from the rectosigmoid to the anus. Approximately one third of all colorectal cancers constitutes of rectal cancer.

CRC originates most often sporadically, is inherited in only 5% of the cases and evolves from benign pre-neoplastic lesions, such as adenomatous polyps or adenomas. The adenoma-carcinoma sequence, a series of well-defined histological stages, is responsible for progression of these benign lesions to malignant carcinomas [5]. Hanahan and Weinberg established six biological capabilities which tumors must acquire during the multistep development of human cancers, also called the hallmarks of cancer [6]. These hallmarks are sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death. More recently, they added two emerging hallmarks; reprogramming of energy metabolism and evading immune recognition and recognized the importance of the tumor-microenvironment, a repertoire of recruited normal cells around the tumor that contributes to the acquisition of these hallmarks as well [7].

Underlying these hallmarks are genome instability, which generates the genetic diversity responsible for the acquisition of these hallmarks, and inflammation [7]. In CRC, three major mechanisms of genetic instability responsible for tumor development and progression have been identified (Figure 1). The first mechanism is through mutations in DNA mismatch repair (MMR) genes, which results in a failure to repair errors that occur during DNA replication, followed by alteration of the length of short, repetitive DNA sequences, called microsatellites, that occur in the human genome. This failure leads to the microsatellite instability (MSI) phenotype and is the hallmark of the hereditary Lynch Syndrome [8]. In addition, in 12-15% of sporadic CRCs MSI has been found as well, but here hypermethylation of the *hMLH1* promotor has been associated with this MSI phenotype [8]. MSI tumors are more frequently right-sided, poorly differentiated, display more often the mucinous cell-type, show more peritumoral lymphocytic infiltration and are associated with an improved survival [9]. Second, most CRCs arise through the chromosomal instability (CIN) pathway, which is also involved in CRC pathogenesis. CIN is observed in 65-70% of sporadic CRC and is characterized by allelic losses (loss of heterozygosity), chromosomal amplifications, and translocations in CRC cells [9;10]. More recently, the existence of a new pathway for CRC development has gained attention.

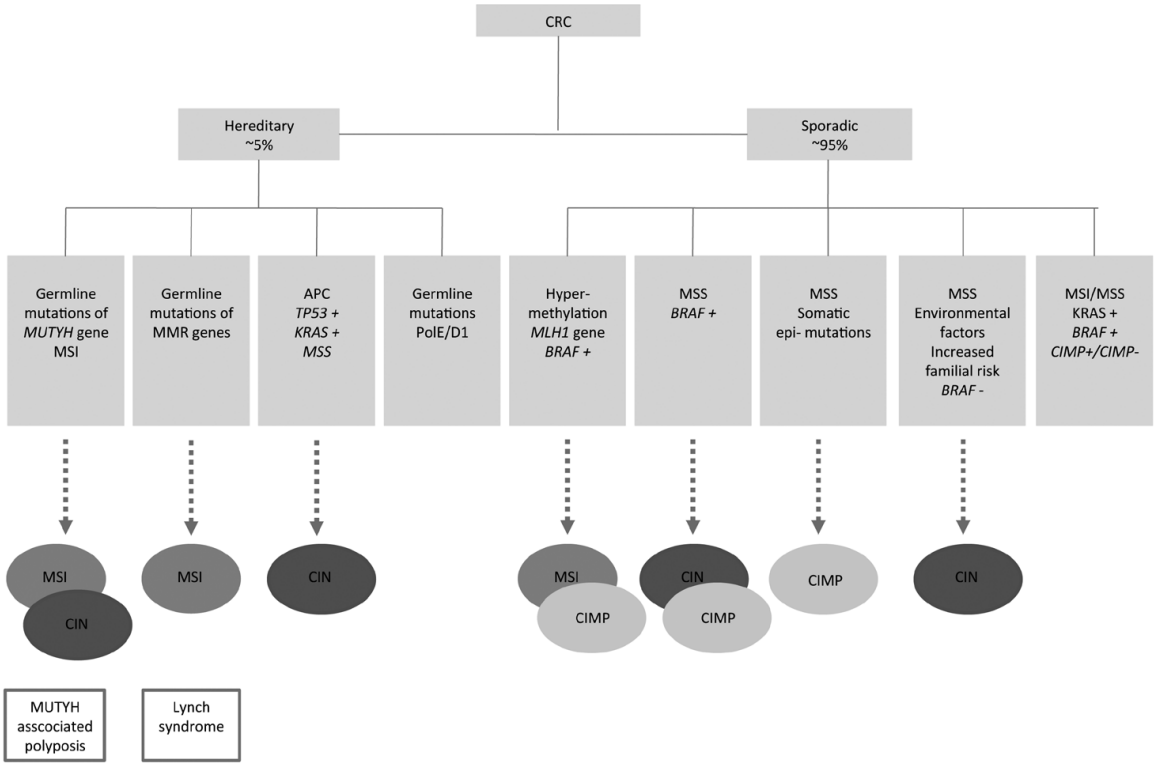


Figure 1: Global overview of major mechanisms of genetic instability responsible for tumor development and progression in CRC.
Abbreviations; CRC colorectal cancer, MSI microsatellite instability, MSS microsatellite stability, CIN chromosomal instability, CIMP CpG island methylator phenotype, BER base excision repair machinery, MMR mismatch repair, MLH1 MutL homolog 1, APC adenomatous polyposis coli, TP53 tumor protein 53, KRAS V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, MUTYH mutY Homolog, PolE DNA polymerase ε, PolD1 DNA polymerase δ. + indicates mutation, - indicates wildtype.

These tumors are classified as having the CpG island methylator phenotype (CIMP), which involves the transcriptional silencing of tumor suppressor genes by hypermethylation of CpG islands of the promoter region of various genes [11]. Approximately one-third to one-half of all CRCs may evolve through this pathway [12].

Recently, three main molecularly distinct subtypes of colon cancer, each associated with unique clinical and molecular features, were demonstrated [13]. The first subtype demarcates the well characterized MSI/CIMP+ subset of colon cancers, which is mainly located on the right side of the colon. The second subtype, mostly left-sided, is largely devoid of MSI/CIMP+, but is typically associated with *KRAS* and/or *TP53* mutations, suggesting to represent the well-described CIN tumors. The third subtype is evenly distributed throughout the colon, enriched with histologically poorly differentiated tumors, heterogeneous with respect to MSI or MSS and CIMP status, and contains a large proportion of *KRAS* and *BRAF* mutations as well. This subtype is associated with a poor prognosis and poor response to anti-epidermal growth factor receptor (EGFR) therapy. The enhanced expression of epithelial mesenchymal transition (EMT) and matrix remod-

eling in these tumors provides a possible explanation for their poor prognosis and why these tumors metastasize more frequently as compared to subtype 2. Furthermore, evidence showed that this subtype is highly related to serrated adenomas as serrated precursor lesions are thought to progress in this subtype of colon cancer [13].

TREATMENT

Treatment choices are nowadays influenced by the tumor, node and metastasis (TNM) classification, which aims to provide an exact prediction system for prognosis, to guide therapy choices and to create uniformity in cancer language [14;15]. The survival of CRC patients largely depends on disease stage at diagnosis and varies widely between stages. In stage I, a five-year survival rate of 93.6% is seen, which drastically drops to 8.1% in stage IV patients [16].

The treatment of colon cancer comprises surgical resection of the primary tumor and regional lymph nodes. The last two decades, the role of adjuvant chemotherapy has gained importance and resulted in the introduction of a chemotherapy regimen in the Netherlands, consisting of oxaliplatin, fluorouracil and leucovorin, in stage III (lymph positive) and high-risk stage II colon cancer patients [17].

Nowadays, patients with rectal cancer are treated with pre-operative (chemo) radiation (5x5 Gy) followed by surgical resection using the total mesorectal excision (TME) technique. Before the introduction of the TME technique the 5-year local recurrence rate of rectal cancer with conventional surgery was over 20% [18]. The last decades these local recurrence rates have decreased drastically, mainly influenced by the introduction of the TME technique and the introduction of pre-operative radiotherapy since the Dutch TME trial, which investigated the effect of short-term preoperative radiotherapy in combination with TME surgery compared to TME surgery alone between 1996 and 2000 [19;20]. The role of adjuvant chemotherapy in rectal cancer is still debatable. The use of adjuvant chemotherapy in patients not treated with pre-operative radiotherapy or chemotherapy seems beneficial [21], however, in patients treated pre-operatively no survival benefit has been reported [22-24].

ASPIRIN TREATMENT

The last decade aspirin is gaining ground in the treatment of CRC patients. There is a significant amount of evidence demonstrating that aspirin has anti-cancer effects [25-32]. The first evidence comes from large cardiovascular prevention trials assessing the cardiovascular benefits of aspirin [29-32]. In a pooled analysis of five large trials aspirin

taken for several years at doses of at least 75 mg daily has shown to reduce long-term incidence and mortality due to CRC [29]. Furthermore, aspirin showed to significantly reduce adenoma formation in patients with a history of CRC [33]. More recently, aspirin has shown to be beneficial as adjuvant treatment as well. Aspirin taken after diagnosis significantly improved overall survival and colorectal cancer-specific mortality in patients with CRC [26;28;34]. At the moment, three recently started trials, ASCOLT in Asian CRC patients, the Big A trial in lung cancer patients and the Add Aspirin trial in colorectal-, breast-, upper gastrointestinal- and prostate – cancer patients, investigate the role of aspirin as adjuvant treatment (http://clinicaltrials.gov).

The exact mechanism by which aspirin exerts its activity is not completely understood. Direct inhibition of the cyclooxygenase (COX) family of enzymes involved in prostaglandin synthesis has been attributed to the protective activity of aspirin. The COX-2 enzyme is strongly and rapidly induced in response to mediators of inflammation, growth factors, cytokines, and endotoxins; and its expression correlates with increased cell proliferation and tumor promotion [35]. Aspirin can decrease the production of potentially neoplastic prostaglandins arising from COX-2 mediated catalysis of arachidonic acid [36].

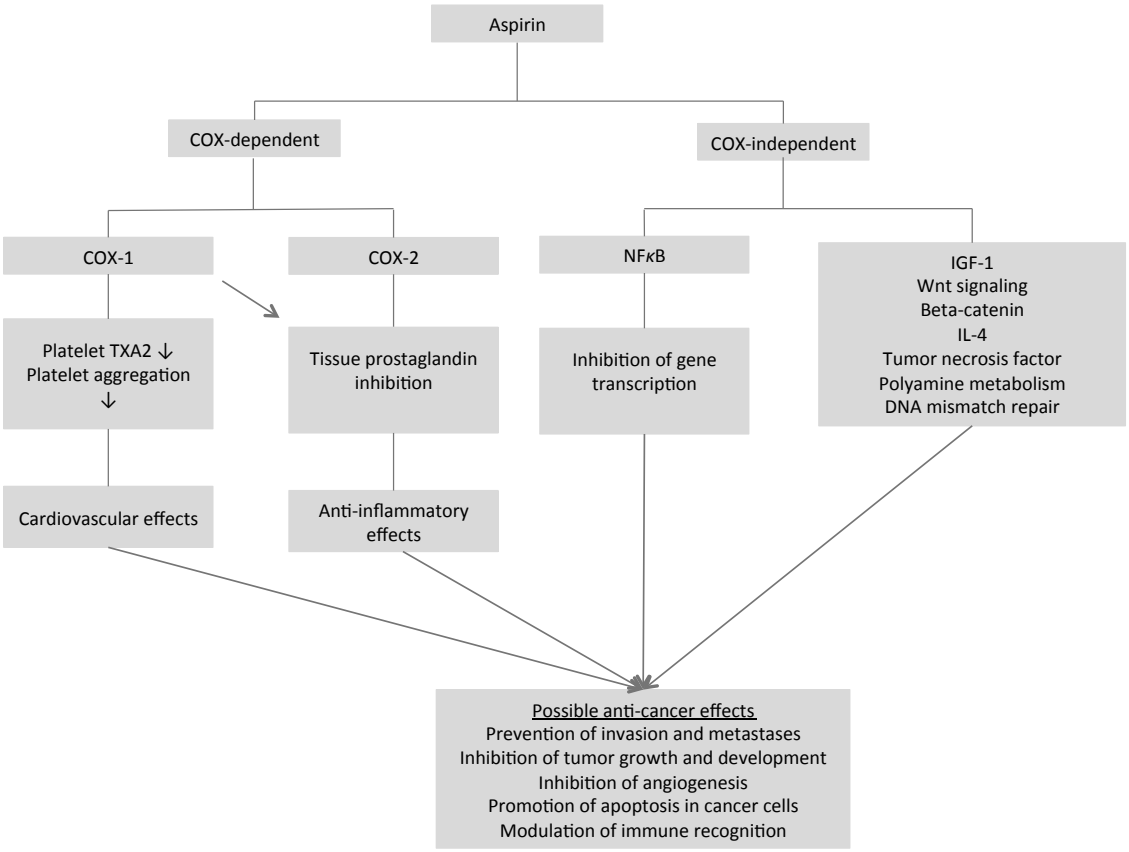


Figure 2: Global overview of possible pathways responsible for the anti-cancer effects of aspirin.
Abbreviations; COX: cyclooxygenase,TXA2 thromboxane A2, NFκB nuclear factor-κB, IGF-1 insulin growth factor 1. Partly based on Langley *et al*, BJC 2011; 105,1107-1113.

However, research has shown that aspirin has a much broader range of downstream effectors as well, such as NF-κB, insulin-like growth factor I (IGF-1), and the inhibition of Wnt-signaling and stem cell growth possibly as the result of enhanced beta-catenin phosphorylation [27;37-39] (Figure 2).

Studies trying to unravel the anti-cancer effects of aspirin thus far have been inconsistent. Possibly, more than one mechanism is responsible for the anti-cancer effects of aspirin. It is also plausible that different molecular mechanisms are responsible for the beneficial effects of aspirin on CRC incidence (prevention) than on already established CRC (therapy).

In the preventive setting of CRC COX-2 might play an influential role since regular aspirin use has shown to be associated with a lower risk of CRCs that overexpress COX-2, but not CRCs without COX-2 overexpression [26]. Also, inhibition of WNT/cadherin-associated protein β1 signaling (CTNNB1 or β-catenin), one of the most essential oncogenic pathways in CRC, has been described to reduce the risk for CRC. Aspirin inhibits this CTNNB1 signaling pathway COX-dependently but also through COX-independent pathways by directly inducing phosphorylation and subsequent degradation of CTNNB1 [40]. More recently, a study showed that aspirin use stabilizes DNA methylation at promotors of genes controlling critical cancer pathways. Age dependent methylation was suppressed in aspirin users and long-term aspirin use was associated with a more than 50% suppressed rate of methylation when compared with nonuse. Aberrant DNA methylation in gene promotors has been associated with aging and cancer [41].

In the first study investigating the molecular mechanisms responsible for the therapeutic effect of aspirin after a CRC diagnosis, COX-2 expression was mentioned to play a major role [26]. The survival benefit with aspirin use after diagnosis in CRC was associated with COX-2 expression of the tumor. A much lower risk of CRC-specific and overall mortality with tumors that overexpress COX-2 was found.

Research from the same group has also shown that aspirin may suppress cancer-cell growth and induce apoptosis by blocking the phosphatidylinositol 3-kinase (PI3K) pathway upstream of COX-2 [42]. This pathway plays an important role in carcinogenesis [43]. Mutations in *PIK3CA* (gene encoding for phosphatidylinositol-4,5-biphosphonate 3-kinase, catalytic subunit alpha polypeptide) are found in approximately 15 to 20% of colorectal cancers [44].

Furthermore, COX-1 might also be responsible for the improved survival of aspirin users in CRC, since aspirin influences platelet aggregation through COX-1 inhibition [45]. Recent evidence suggest that platelets may play an important active role in promoting metastasis by active signalling to tumor cells through the TGF-β and NF-κB pathways resulting in a prometastatic phenotype that facilitates tumor cell extravasation and metastasis [46]. Aspirin has shown to inhibit the activation of NF-κB [47].

Finally, it has been shown that IL-4 expression is essential for the resistance to DNA damage-induced apoptosis of colon cancer stem cells (CSCs) [48]. CSCs are also resistant to the cytotoxic effect of chemotherapy. It has been shown that IL-4 confers colon CSCs with resistance to apoptosis [48]. Consistently, treatment with IL-4Ra antagonist or anti-IL-4 neutralizing antibody strongly enhances the antitumor efficacy of standard chemotherapeutic drugs through selective CSCs sensitization. Notably, aspirin inhibits IL-4 gene expression [49]. Based on the above observations, it is plausible that aspirin may both act as a preventive agent in CRC onset by modulating the Wnt pathway in CSCs, but also as adjuvant treatment by increasing CSCs' sensitivity to conventional chemotherapy regimens.

PROGNOSTIC AND PREDICTIVE BIOMARKERS IN CRC

To date, tumor location and tumor stage have majorly influenced treatment decisions. However, new insights and advances in the molecular biology of CRC have started to influence prognostication and treatment decisions. Molecular mechanisms responsible for tumorigenesis are likely to influence clinical outcome [6]. Also, research has shown that approximately 20-25% of patients with lymph-node negative stage II colon cancer, which were not recommended adjuvant treatment based on TNM stage, suffer from recurrent disease within 5-years of follow-up [50]. The TNM stage is therefore not an optimal tool for prognostication and treatment allocation, especially in high-risk stage II patients, and needs to be supplemented with additional biomarkers that can improve the current staging and treatment allocation criteria substantially.

By investigating biomarkers that reflect tumor growth and metastatic potential, a more accurate prediction on prognosis and treatment benefit based on underlying biology can be made. Predicting the clinical behavior of a tumor through a combination of clinical, pathological and biological characteristics may lead to a well-targeted treatment in the individual patient. Evading immune recognition, sustaining proliferative signaling and resisting cell death are important mechanisms that cancer cells acquire during further tumor development [7] and are therefore studied in the research described in this thesis.

OUTLINE OF THIS THESIS

The aim of this thesis was to define prognostic and predictive biomarkers in colorectal cancer for improved risk stratification and treatment benefit in the individual patient, with the introduction of precision medicine in the near future as the ultimate goal. By

definition, precision medicine is a multi-faceted approach to medicine that integrates molecular and clinical research with patient data and clinical outcome, and places the patient at the center of all elements. This thesis is divided in three parts. In ***Part one*** prognostic biomarkers in CRC are investigated, in ***Part two*** aspirin treatment and related predictive biomarkers for aspirin treatment benefit in colon cancer are investigated and finally, in ***Part three***, the use of predictive and prognostic biomarkers in clinical practice, its utility and the road to precision medicine are discussed.

The last two decades, research has shown that the immune system has a substantial effect on tumor growth and metastasis [51]. Tumors are thought to be 'edited' through a Darwinian selection process in poorly immunogenic tumor cell variants able to evade immune recognition and consequently growth progression [52-55]. Several mechanisms in the tumor contribute to this process. First, downregulation of human leukocyte antigen (HLA) class I expression, which minimizes the level of tumor-associated antigen (TAA) expression by tumor cells, followed by less immune recognition and subsequently less destruction by cytotoxic T-cells (CTL) [56]. Second, expression of non-classical HLA class I molecules (HLA-E and HLA-G) on the tumor cell surface. HLA-E is regularly expressed in various healthy tissues and correlates with HLA class I expression [57]. In contrast, HLA-G is rarely expressed in healthy tissues but has been frequently observed in tumors [58]. Both have been associated with inhibition of natural killer (NK) cell recognition resulting in further escape from immune recognition [58;59]. Third, attraction of immunosuppressive regulatory T cells (Tregs) into the tumor micro-environment, which suppress the activity of CTL [60;61]. Conflicting results have been described for the association between expression of these markers and prognosis in CRC patients, possibly due to the use of different patient cohorts and the investigation of solely one marker. Research has shown a complex relationship between different immune markers, highlighting the need for combined marker analysis [62-64]. Therefore, in **Chapter 2** we evaluated the association of these immune markers, separately and combined, with prognosis in colon cancer patients. We performed the same analysis in rectal cancer patients to investigate differences in immune escape mechanisms between colon- and rectal cancer in **Chapter 3.**

Deregulation of the proliferative signaling pathway and deregulation of the apoptotic pathway are also two important hallmarks of tumor development, which disturb tissue homeostasis and balance [6]. Previous studies have shown contradicting results with respect to the relation of apoptotic - or proliferation levels in tumor specimens and patient outcome in CRC [65-68]. In **Chapter 4** we therefore investigated if the combined analysis of these two processes would better reflect tumor aggressiveness.

Over the last decades the public health sector witnessed a vast and rapid development of genomic profiling techniques, with the promise of precision medicine as a strong driving force. Prediction of pathway deregulation coupled to molecular target identification using genome wide approaches may provide an opportunity to guide

treatment [69]. Since various molecular pathways are involved in carcinogenesis, multigene assays might give a more reliable insight in tumour biology and risk of recurrence than single-gene analysis. One of those multi-gene assays is the Onco*type* DX Colon Cancer Recurrence Score (RS) (Genomic Health, Redwood City, CA, USA), which measures the expression of 12 genes and was validated as a predictor of recurrence risk in stage II colon cancer patients [70;71]. Validation of this multi-gene assay in rectal cancer has been performed and described in **Chapter 5.**

In ***Part two*** of this thesis, the benefit from aspirin treatment in colon cancer is described. In **Chapter 6**, this benefit was investigated in older colon cancer patients. Recent studies have shown that regular use of aspirin after diagnosis was associated with longer survival among patients with mutated- *PIK3CA* CRC, but not among wild-type *PIK3CA* tumors [44], and among patients who express high tumor levels of COX-2 [26]. In **Chapter 7** we showed that colon cancer patients only benefit from aspirin treatment when these patients expressed HLA class I on their tumor cell surface. The aspirin benefit on survival was not associated with *PIK3CA* or COX-2 expression in our cohort.

In ***Part three*** of this thesis the use and introduction of biomarkers in clinical practice influencing precision therapy (**Chapter 8**) and the impact of genomic profiling on surgery (**Chapter 9**) are discussed. Finally, an overall summary and discussion of the data presented in this thesis are provided in **Chapter 10**.

REFERENCE LIST

(1) Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010;127:2893-2917.

(2) Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011;61:69-90.

(3) La Vecchia C., Bosetti C, Lucchini F et al. Cancer mortality in Europe, 2000-2004, and an overview of trends since 1975. *Ann Oncol* 2010;21:1323-1360.

(4) Malvezzi M, Arfe A, Bertuccio P, Levi F, La VC, Negri E. European cancer mortality predictions for the year 2011. *Ann Oncol* 2011;22:947-956.

(5) Vogelstein B, Fearon ER, Hamilton SR et al. Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988;319:525-532.

(6) Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57-70.

(7) Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646-674.

(8) Markowitz SD, Bertagnolli MM. Molecular origins of cancer: Molecular basis of colorectal cancer. *N Engl J Med* 2009;361:2449-2460.

(9) Gryfe R, Kim H, Hsieh ET et al. Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer. *N Engl J Med* 2000;342:69-77.

(10) Pino MS, Chung DC. The chromosomal instability pathway in colon cancer. *Gastroenterology* 2010;138:2059-2072.

(11) Hawkins N, Norrie M, Cheong K et al. CpG island methylation in sporadic colorectal cancers and its relationship to microsatellite instability. *Gastroenterology* 2002;122:1376-1387.

(12) Goel A, Arnold CN, Niedzwiecki D et al. Characterization of sporadic colon cancer by patterns of genomic instability. *Cancer Res* 2003;63:1608-1614.

(13) De Sousa E Melo, Wang X, Jansen M et al. Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. *Nat Med* 2013;19:614-618.

(14) Gospodarowicz MK, Miller D, Groome PA, Greene FL, Logan PA, Sobin LH. The process for continuous improvement of the TNM classification. *Cancer* 2004;100:1-5.

(15) Greene FL, Sobin LH. The staging of cancer: a retrospective and prospective appraisal. *CA Cancer J Clin* 2008;58:180-190.

(16) O'Connell JB, Maggard MA, Ko CY. Colon cancer survival rates with the new American Joint Committee on Cancer sixth edition staging. *J Natl Cancer Inst* 2004;96:1420-1425.

(17) Andre T, Boni C, Mounedji-Boudiaf L et al. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. *N Engl J Med* 2004;350:2343-2351.

(18) Kapiteijn E, Marijnen CA, Colenbrander AC et al. Local recurrence in patients with rectal cancer diagnosed between 1988 and 1992: a population-based study in the west Netherlands. *Eur J Surg Oncol* 1998;24:528-535.

(19) Kapiteijn E, Marijnen CA, Nagtegaal ID et al. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. *N Engl J Med* 2001;345:638-646.

(20) van Gijn W, Marijnen CA, Nagtegaal ID et al. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer: 12-year follow-up of the multicentre, randomised controlled TME trial. *Lancet Oncol* 2011;12:575-582.

(21) Petersen SH, Harling H, Kirkeby LT, Wille-Jorgensen P, Mocellin S. Postoperative adjuvant chemotherapy in rectal cancer operated for cure. *Cochrane Database Syst Rev* 2012;3:CD004078.

(22) Bosset JF, Collette L, Calais G et al. Chemotherapy with preoperative radiotherapy in rectal cancer. *N Engl J Med* 2006;355:1114-1123.

(23) Bujko K, Glynne-Jones R, Bujko M. Adjuvant chemotherapy for rectal cancer. *Ann Oncol* 2010;21: 2443.
(24) Glimelius B, Dahl O, Cedermark B et al. Adjuvant chemotherapy in colorectal cancer: a joint analysis of randomised trials by the Nordic Gastrointestinal Tumour Adjuvant Therapy Group. *Acta Oncol* 2005;44:904-912.
(25) Algra AM, Rothwell PM. Effects of regular aspirin on long-term cancer incidence and metastasis: a systematic comparison of evidence from observational studies versus randomised trials. *Lancet Oncol* 2012;13:518-527.
(26) Chan AT, Ogino S, Fuchs CS. Aspirin use and survival after diagnosis of colorectal cancer. *JAMA* 2009;302:649-658.
(27) Langley RE, Burdett S, Tierney JF, Cafferty F, Parmar MK, Venning G. Aspirin and cancer: has aspirin been overlooked as an adjuvant therapy? *Br J Cancer* 2011;105:1107-1113.
(28) McCowan C, Munro AJ, Donnan PT, Steele RJ. Use of aspirin post-diagnosis in a cohort of patients with colorectal cancer and its association with all-cause and colorectal cancer specific mortality. *Eur J Cancer* 2013;49:1049-1057.
(29) Rothwell PM, Wilson M, Elwin CE et al. Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomised trials. *Lancet* 2010;376:1741-1750.
(30) Rothwell PM, Fowkes FG, Belch JF, Ogawa H, Warlow CP, Meade TW. Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. *Lancet* 2011;377:31-41.
(31) Rothwell PM, Wilson M, Price JF, Belch JF, Meade TW, Mehta Z. Effect of daily aspirin on risk of cancer metastasis: a study of incident cancers during randomised controlled trials. *Lancet* 2012; 379:1591-1601.
(32) Rothwell PM, Price JF, Fowkes FG et al. Short-term effects of daily aspirin on cancer incidence, mortality, and non-vascular death: analysis of the time course of risks and benefits in 51 randomised controlled trials. *Lancet* 2012.
(33) Cole BF, Logan RF, Halabi S et al. Aspirin for the chemoprevention of colorectal adenomas: meta-analysis of the randomized trials. *J Natl Cancer Inst* 2009;101:256-266.
(34) Bastiaannet E, Sampieri K, Dekkers OM et al. Use of Aspirin postdiagnosis improves survival for colon cancer patients. *Br J Cancer* 2012.
(35) Herschman HR. Prostaglandin synthase 2. *Biochim Biophys Acta* 1996;1299:125-140.
(36) Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol* 1971;231:232-235.
(37) Dihlmann S, Siermann A, von Knebel DM. The nonsteroidal anti-inflammatory drugs aspirin and indomethacin attenuate beta-catenin/TCF-4 signaling. *Oncogene* 2001;20:645-653.
(38) Dihlmann S, Klein S, Doeberitz MM. Reduction of beta-catenin/T-cell transcription factor signaling by aspirin and indomethacin is caused by an increased stabilization of phosphorylated beta-catenin. *Mol Cancer Ther* 2003;2:509-516.
(39) Wang Y, Chen X, Zhu W, Zhang H, Hu S, Cong X. Growth inhibition of mesenchymal stem cells by aspirin: involvement of the WNT/beta-catenin signal pathway. *Clin Exp Pharmacol Physiol* 2006;33: 696-701.
(40) Nan H, Morikawa T, Suuriniemi M et al. Aspirin use, 8q24 single nucleotide polymorphism rs6983267, and colorectal cancer according to CTNNB1 alterations. *J Natl Cancer Inst* 2013;105: 1852-1861.
(41) Noreen F, Roosli M, Gaj P et al. Modulation of age- and cancer-associated DNA methylation change in the healthy colon by aspirin and lifestyle. *J Natl Cancer Inst* 2014;106.

(42) Uddin S, Ahmed M, Hussain A et al. Cyclooxygenase-2 inhibition inhibits PI3K/AKT kinase activity in epithelial ovarian cancer. *Int J Cancer* 2010;126:382-394.
(43) Samuels Y, Wang Z, Bardelli A et al. High frequency of mutations of the PIK3CA gene in human cancers. *Science* 2004;304:554.
(44) Liao X, Lochhead P, Nishihara R et al. Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. *N Engl J Med* 2012;367:1596-1606.
(45) Ofosu FA. Effective, selective and specific inhibition of COX-1 may overcome the "aspirin paradox". *Thromb Res* 2013;132:1-2.
(46) Labelle M, Begum S, Hynes RO. Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. *Cancer Cell* 2011;20:576-590.
(47) Kopp E, Ghosh S. Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* 1994;265: 956-959.
(48) Todaro M, Alea MP, Di Stefano AB et al. Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. *Cell Stem Cell* 2007;1:389-402.
(49) Cianferoni A, Schroeder JT, Kim J et al. Selective inhibition of interleukin-4 gene expression in human T cells by aspirin. *Blood* 2001;97:1742-1749.
(50) Davies M, Arumugam PJ, Shah VI et al. The clinical significance of lymph node micrometastasis in stage I and stage II colorectal cancer. *Clin Transl Oncol* 2008;10:175-179.
(51) Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 2011;331:1565-1570.
(52) Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* 2002;3:991-998.
(53) Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* 2004;21:137-148.
(54) Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. *Annu Rev Immunol* 2004;22: 329-360.
(55) Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol* 2006;6:836-848.
(56) Cavallo F, De GC, Nanni P, Forni G, Lollini PL. 2011: the immune hallmarks of cancer. *Cancer Immunol Immunother* 2011;60:319-326.
(57) Palmisano GL, Contardi E, Morabito A, Gargaglione V, Ferrara GB, Pistillo MP. HLA-E surface expression is independent of the availability of HLA class I signal sequence-derived peptides in human tumor cell lines. *Hum Immunol* 2005;66:1-12.
(58) Wischhusen J, Waschbisch A, Wiendl H. Immune-refractory cancers and their little helpers—an extended role for immunetolerogenic MHC molecules HLA-G and HLA-E? *Semin Cancer Biol* 2007; 17:459-468.
(59) Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nat Immunol* 2002;3:999-1005.
(60) Nosho K, Baba Y, Tanaka N et al. Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer, and prognosis: cohort study and literature review. *J Pathol* 2010;222:350-366.
(61) Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat Rev Immunol* 2006;6:715-727.
(62) de Kruijf EM, van Nes JG, Sajet A et al. The predictive value of HLA class I tumor cell expression and presence of intratumoral Tregs for chemotherapy in patients with early breast cancer. *Clin Cancer Res* 2010;16:1272-1280.

(63) de Kruijf EM, Sajet A, van Nes JG et al. HLA-E and HLA-G expression in classical HLA class I-negative tumors is of prognostic value for clinical outcome of early breast cancer patients. *J Immunol* 2010; 185:7452-7459.

(64) Zeestraten EC, Van Hoesel AQ, Speetjens FM et al. FoxP3- and CD8-positive Infiltrating Immune Cells Together Determine Clinical Outcome in Colorectal Cancer. *Cancer Microenviron* 2011.

(65) de Bruin EC, van de Velde CJ, van de Pas S et al. Prognostic value of apoptosis in rectal cancer patients of the dutch total mesorectal excision trial: radiotherapy is redundant in intrinsically high-apoptotic tumors. *Clin Cancer Res* 2006;12:6432-6436.

(66) de Heer P, de Bruin EC, Klein-Kranenbarg E et al. Caspase-3 activity predicts local recurrence in rectal cancer. *Clin Cancer Res* 2007;13:5810-5815.

(67) Fluge O, Gravdal K, Carlsen E et al. Expression of EZH2 and Ki-67 in colorectal cancer and associations with treatment response and prognosis. *Br J Cancer* 2009;101:1282-1289.

(68) Jonges LE, Nagelkerke JF, Ensink NG et al. Caspase-3 activity as a prognostic factor in colorectal carcinoma. *Lab Invest* 2001;81:681-688.

(69) Bild AH, Yao G, Chang JT et al. Oncogenic pathway signatures in human cancers as a guide to targeted therapies. *Nature* 2006;439:353-357.

(70) Gray RG, Quirke P, Handley K et al. Validation study of a quantitative multigene reverse transcriptase-polymerase chain reaction assay for assessment of recurrence risk in patients with stage II colon cancer. *J Clin Oncol* 2011;29:4611-4619.

(71) Venook AP, Niedzwiecki D, Lopatin M et al. Biologic determinants of tumor recurrence in stage II colon cancer: validation study of the 12-gene recurrence score in cancer and leukemia group B (CALGB) 9581. *J Clin Oncol* 2013;31:1775-1781.

PART ONE

Prognostic biomarkers in colorectal cancer

CHAPTER 2

Combined analysis of HLA Class I, HLA-E and HLA-G predicts prognosis in colon cancer patients

Marlies S. Reimers*, Eliane C.M. Zeestraten*, Sepideh Saadatmand, Jan-Willem T. Dekker, Gerrit Jan Liefers, Peter J.van den Elsen, Cornelis J.H. van de Velde, Peter J.K. Kuppen

** Both authors contributed equally to this work*

British Journal of Cancer, 2014 Jan 21; 110(2): 459-68

ABSTRACT

Background

Evasion of immune surveillance and suppression of the immune system are important hallmarks of tumor development in colon cancer. The goal of this study was to establish a tumor profile based on biomarkers that reflect a tumors' immune susceptibility status and to determine their relation to patient outcome.

Methods

The study population consisted of 285 Stage I-IV colon cancer patients of which a tissue micro array (TMA) was available. Sections were immuno-histochemically stained for presence of Foxp3+ cells and tumor expression of HLA Class I (HLA-A, -B, -C) and non-classical HLA-E and HLA-G. All markers were combined for further analyses, resulting in 3 tumor immune phenotypes: a, strong immune system tumor recognition; b, intermediate immune system tumor recognition; and c, poor immune system tumor recognition.

Results

Loss of HLA class I expression was significantly related to a better OS (p-value 0.005) and DFS (p-value 0.008). Patients with tumors that showed neither HLA class I nor HLA-E or -G expression (phenotype a) had a significant better OS and DFS (*p*-value <0.001 and 0.001, respectively) compared to phenotype b (OS HR 4.7, 95% CI 1.2-19.0, *p*=0.001) or c (OS HR 8.2, 95% CI 2.0-34.2, *p*=0.0001). Furthermore, the tumor immune phenotype was an independent predictor for OS and DFS (*p*=0.009 and 0.013 respectively).

Conclusions

Tumors showing absence of HLA class I, HLA-E and HLA-G expression were related to a better OS and DFS. By combining the expression status of several immune-related biomarkers, three tumor immune phenotypes were created that related to patient outcome. These immune phenotypes represented significant, independent, clinical prognostic profiles in colon cancer.

INTRODUCTION

Historically, the immune system has been attributed an important role in controlling tumor growth and metastasis [1-4]. Evasion of immune surveillance and suppression of the immune system are two important traits cancer cells have to acquire during the process of tumorigenesis [5]. Research of the last century has indicated that the influence of the immune system on tumor cells, both in the tumor micro-environment as well as during the process of tumor metastasis, also contributes to tumor progression [6]. The cancer immune-editing hypothesis describes both the host-protective as well as the tumor-promoting actions the immune system might have on developing tumors, shaping tumor immunogenicity [7-13]. Tumors are thought to be 'edited' through a Darwinian selection process into poorly immunogenic tumor cell variants invisible to the immune system and able to grow progressively. Immune-editing might therefore have substantial effects on patient's prognosis.

Several mechanisms taking place at the tumor cell level contribute to this process. The first mechanism is downregulation of human leukocyte antigen (HLA) class I expression. Downregulation of HLA class I minimizes the level of tumor-associated antigen (TAA) expression by tumor cells and therefore their recognition and subsequently destruction by cytotoxic T-cells (CTL) [5;14-16]. The second mechanism is the ability of tumor cells to regulate the expression of non-classical HLA class I molecules (HLA-E and HLA-G) on the cell surface. Expression of these markers has been found to inhibit Natural Killer (NK) cell recognition in the blood stream and therefore results in further tumor cell escape from immune surveillance [17-20]. HLA-E is regularly expressed in various healthy tissues and correlated with HLA class I expression [21]. In contrast, HLA-G is rarely found in healthy tissues, but is frequently observed in tumors [19]. Thirdly, tumor cell immune reactivity can become suppressed by the attraction of immunosuppressive regulatory T cells (Tregs) into the tumor micro-environment [22;23]. Tregs are able to modulate the anti-tumor immune response as they suppress the activity of CTL through direct cell-to-cell contact or via the release of cytokines like transforming growth factor β [24-26]. Tregs and CTLs therefore show opposing actions in tumor immunity [27].

Previously, both the downregulation of HLA class I, presence of Tregs and HLA-E and -G expression have been shown to be of clinical relevance in several types of cancers [28-31]. In colorectal cancer (CRC), various studies have described the impact of the level of HLA class I tumor expression or the presence of Foxp3+ Tregs cells on patients with varying results [32-39]. In general, loss of HLA class I tumor expression seemed to result in a better prognosis [39;40]. The presence of high levels of Foxp3+ cells in CRC patients was related to a worse prognosis in some studies, although this relation could not always be established in CRC patients [33;34;37;38;41]. Studies on the prognostic value of HLA-E and

HLA-G showed that expression of these molecules correlated with poor prognosis and tumor progression [42-45].

Previous studies have shown a complex interaction between different immune markers, highlighting the need for combined marker analysis [29;41;46]. The purpose of this study was to investigate the prognostic value of the immune-related biomarkers HLA Class I, HLA-E and -G and Foxp3+, to establish distinct patterns that reflect a tumor's immune-escape mechanism by combining these markers, and to relate these patterns to clinical outcome.

MATERIALS AND METHODS

Study population

The patient population comprised a consecutive series of 470 colorectal cancer patients all treated with surgery for their primary tumor in the Leiden University Medical Center (LUMC) between 1991 and 2001. Of these patients tumor material, clinico-pathological data and information on the follow-up was collected in retrospect. This research was performed according to the code of conduct for responsible use. Mucinous differentiation was defined as fully (>50%), partly (0-50%) or no mucinous differentiation. Tumor Node Metastasis (TNM) was defined by the Union for International Cancer Control (UICC) [47]. Tumor differentiation was defined as good, moderate or poor, as described in the pathology report. Patients with rectal cancer, patients with a history of cancer other than basal cell carcinoma or cervical carcinoma *in situ*, patients with more than one colon tumor at the same time, and patients that received radio- or chemotherapy treatment prior to resection were excluded from the analysis (n=185 in total). The study cohort therefore consisted of 285 colon cancer patients.

Antibodies

The mouse monoclonal antibodies HCA2 and HC10 were used, which recognize the heavy chains of HLA Class I, and were kindly provided by Prof. Dr. J. Neefjes. The reactivity spectrum of HCA2 comprises all HLA-A chains (except HLA-A24), as well as some HLA-B, HLA-C, HLA-E, HLA-F, and HLA-G chains. HC10 reacts with HLA-B and HLA-C heavy chains and some HLA-A (HLA-A10, HLA-A28, HLA-A29, HLA-A30, HLA-A31, HLA-A32, HLA-A33) [46]. The mouse antibodies against human Foxp3 (ab20034 clone 236A/E7; Abcam) were used for Treg identification. The reactivity spectrum of Foxp3 is composed of regulatory T cells and may include small numbers of CD8+ cells but is generally considered to be the best single marker for Treg identification [48;49]. For HLA-E and HLA-G identification mouse monoclonal antibodies against HLA-E (ab2216 clone MEM-E/02: AbCam, UK) and HLA-G (4H84: Exbio, Czech Republic) were used. MEM-E/02 recognizes

denatured HLA-E [50;51], while 4H84 recognizes denatured HLA-G molecules and also binds to free heavy chains of classical HLA class I molecules [51-53].

TMA production and immunohistochemistry

The histo-pathological characteristics of the tumor material from all patients included were determined by qualified pathologists according to current standards. Of the formalin-fixed paraffin-embedded (FFPE) tumor blocks of the primary tumors, sections were cut for haematoxylin and eosin staining. Based on microscopic inspection of the slides, histo-pathologically representative bulk tumor regions from each tumor block were identified and punched for preparation of tumor tissue microarray (TMA) blocks. From each donor block, three 0.6 mm diameter tissue cores were punched from the identified tumor areas and transferred into a receiver paraffin block using a custom-made precision instrument. Immuno-histochemical staining (IHC) for Foxp3+ cells, non-classical HLA-E and HLA-G, and classical HLA class I tumor expression was performed on 4 µm sections, which were cut from each receiver block and mounted on glass.

The sections were deparaffinized and rehydrated according to standard procedures. Endogenous peroxidase was blocked for 20 minutes in 0.3% hydrogen peroxide in PBS. For antigen retrieval, slides were boiled in 0.01 M EDTA buffer (pH 8) for 10 minutes at maximum power in a microwave oven. Sections were incubated overnight with anti-Foxp3+, -HLA-E or -HLA-G antibodies at pre-determined optimal dilution. After 30 minutes of incubation with Envision anti-mouse (K4001; DAKO Cytomation, Glostrup, Denmark), sections were visualized using diaminobenzidine solution (DAB+). Tissue sections were counterstained with haematoxylin, dehydrated and finally mounted in pertex. The IHC for HCA2 and HC10 was performed using the Autostainer Link 48 (DAKO). For antigen retrieval Envision TM Target Retrieval Solution (DAKO), pH low, was used. The sections were incubated for 18 hours with either HCA2 or HC10 antibodies at pre-determined optimal dilution, followed by incubation with Envision FLEX/HRP (DAKO). Sections were visualized using DAB+ liquid solution (DAKO). Finally these slides were counterstained with haematoxylin as well, dehydrated and finally mounted in pertex.

All slides were stained simultaneously to avoid interassay variation. For each patient, normal epithelium, stromal cells, or lymphoid cells served as internal positive control for HLA class I antibody reactivity. Placenta tissue slides served as positive control for HLA-E and HLA-G staining. Slides from human tonsil tissue served as positive control for Foxp3+ staining. Negative controls were tissue slides that did undergo the whole immunohistochemical staining without primary antibody.

Evaluation of immunohistochemistry

Microscopic analysis of HCA2, HC10, HLA-E and HLA-G expression and presence of Foxp3 + cells was performed by two independent observers in a blinded manner (M.S.R.: 100%

of the cohort, E.C.M.Z. 30% of the cohort). The Cohen's Kappa was > 0.75 for all stainings indicating substantial agreement between the two observers. The scores of the three 0.6 mm punches were averaged. For HCA2 and HC10 the percentage of tumor cells with membranous staining was assessed. HLA class I expression status was determined according to the standard set by the International HLA and Immunogenetics Workshop [54]. HCA2 and HC10 expression percentages were divided into two categories; 0-5% of the tumor cells show expression and 5-100% show expression. If <5% of the tumor cells showed expression for each of the two markers, this was determined to represent loss of HLA class I expression; if expression in <5% of the tumor cells of one of the two markers as HLA class 1 downregulation; and if expression in more than 5% of the tumor cells for each of the two markers this was denoted as HLA class I expression. For HLA-E and HLA-G, intensity of tumor staining (absent, weak, moderate or strong intensity) was determined. For HLA-E, absent and weak staining together versus moderate and strong staining together were used for the final analysis. For HLA-G, absent tumor staining was analyzed versus weak, moderate and strong tumor staining together, because HLA-G is normally not expressed on healthy tissues in comparison to HLA-E [19;21]. Quantification of the number of Foxp3+ cells was microscopically assessed in the entire tumor punches of the TMA and the absolute number of positive cells was used for the analysis.

Determination of microsatellite stability status

DNA was extracted from 2mm tumor-cores. Paraffin was dissolved in xylene, tissue was rehydrated in ethanol (100%/70%) and dried for 10 minutes at 37°C. Nucleospin 96 Tissue kit (Machery-Nagel, Düren, Germany) was used for DNA extraction according to the manufacturer's protocol.

MSS-status was tested using the MSI Analysis System Version 1.2 (Promega, Mannheim, Germany) and interpreted by an experienced pathologist, as described previously [55].

Statistical Analysis

Statistical analyses were performed using the statistical package SPSS (version 17.0 for Windows; SPSS Inc.). The Student's T-test and the Chi-squared test were used to evaluate associations between tumor expressions of HLA class I, and non-classical HLA-E and HLA-G and tumor infiltration of Foxp3+ cells and various clinico-pathological variables. Overall Survival (OS) was defined as time of surgery until death and Disease Free Survival (DFS) as time of surgery until death or relapse of disease, whichever came first. The Kaplan-Meier method was used for calculation of survival probabilities and the Log-rank test for comparison of survival curves between these three phenotypes. Cox regression was used for univariate and multivariable analysis for OS and DFS. Significant variables ($p<0.05$) in univariate analysis were included in multivariable analysis.

RESULTS

HLA class I expression

Microscopic quantification of HLA class I expression was performed on 242 patients as, due to staining artifacts and loss of material during the staining procedure, the IHC results of 43 cases could not be analyzed. Representative images of HLA class 1 staining and frequencies of HLA class I expression in the different groups are shown in Figure 1 and 2. Patient characteristics and data on HLA class I expression are shown in

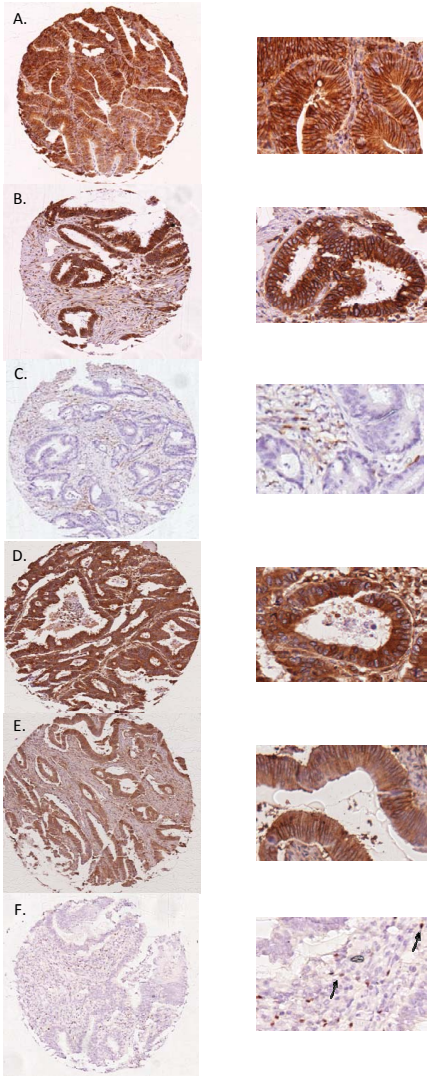


Figure 1: Representative images of HLA class 1, HLA-E, HLA-G and Foxp3+ staining.
Representative images of immunohistochemical stainings for HLA Class I expression (HCA2 and HC10), HLA-E and HLA-G expression and presence of FOXP3+ on the left side with magnifications on the right side, performed according to standard protocols (details in *Material and Methods*).
(A) HCA2-positive tumor (B) HC10-positive tumor (C) HC10- negative tumor with positive internal control (D) HLA-E-positive tumor (E) HLA-G- positive tumor and (F) Presence of Foxp3+ cells as indicated by the arrows.

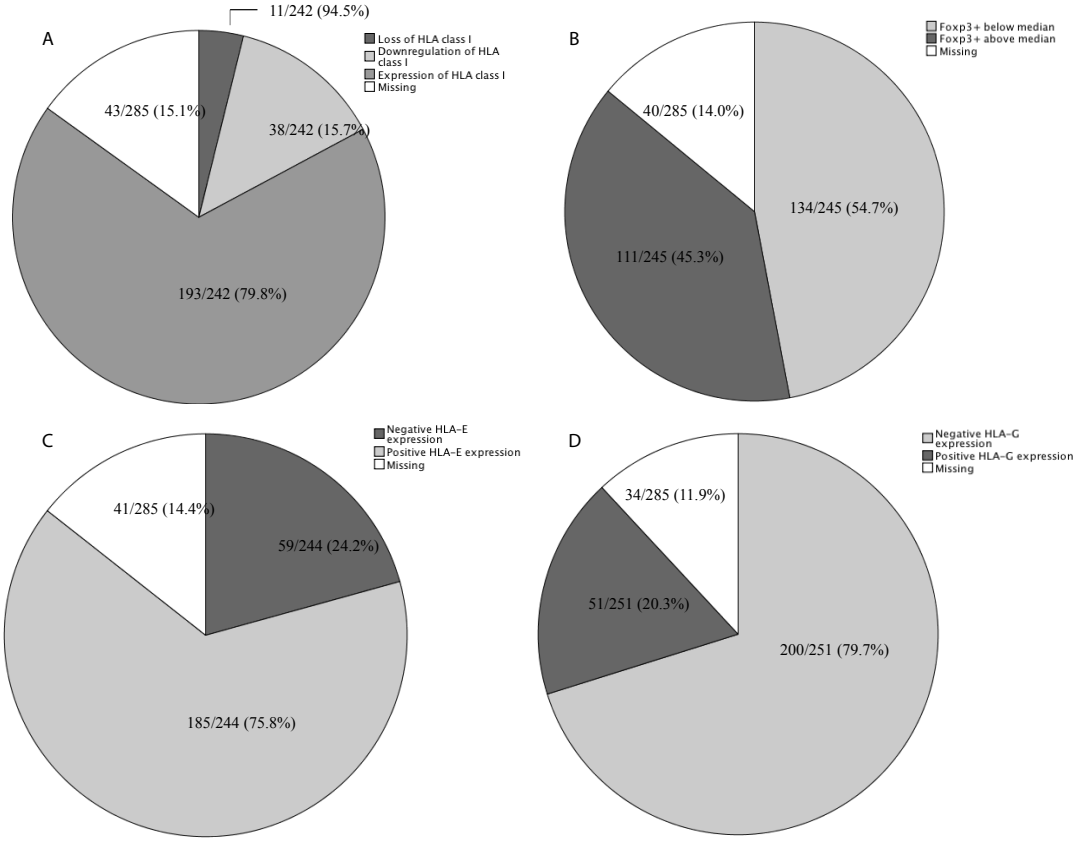


Figure 2: Frequencies of HLA class I tumor expression, Foxp3+ tumor infiltration and HLA-E and –G tumor expression.

Pie-charts indicating the frequencies of all stainings including missings due to staining artifacts and loss during the staining procedure. Details about group composition and scoring methods are written in *Material and Methods.* (A) Frequency of HLA class I tumor expression; (B) Frequency of Foxp3+ tumor cell infiltration; (C) Frequencies of HLA-E tumor expression; (D) Frequency of HLA-G tumor expression.

Table I. Since HCA2 also reacts with some HLA-G chains [46], we examined the relationship between HCA-2 reactivity and HLA-G expression and found no correlation (p=0.348).

Patients whose tumors showed loss of HLA class I had a significantly better OS and DFS (logrank p-value 0.005 and 0.008) compared to patients with tumors with HLA class I downregulation or expression (Figure 3). The Hazard Ratios (HRs) for OS and DFS for HLA class I tumor expression are shown in Table IIa and IIb.

Table I: Patient Characteristics of the Total Colon Cancer Cohort and stratified for HLA class I, HLA-EG and Foxp3+ expression

	Total population (n=285)	HLA Class 1 Loss (n=11)	HLA Class 1 Downregulation (n=38)	HLA Class 1 Expression (n=193)	HLA-EG Absence (n=202)	HLA-EG Presence (n=42)	Foxp3+ Absence (n=134)	Foxp3+ Presence (n=111)
Gender (%)								
Male	137(48.1)	6 (54.5)	18 (47.4)	99 (51.3)	103 (51.0)	17 (41.5)	63 (47.0)	57 (51.4)
Female	148 (51.9)	5 (45.5)	20 (52.6)	94 (48.3)	99 (49.0)	25 (58.5)	71 (53.0)	54 (48.6)
Age in years (%)								
Below 50	32 (11.3)	3 (27.3)	1 (2.6)	23 (12.0)	24 (12.0)	3 (7.1)	13 (9.7)	14 (12.8)
Above 50	251 (88.7)	8 (72.9)	37 (97.4)	168 (88.0)	176 (88.0)	39 (92.9)	121 (90.3)	95 (87.2)
T stage (%)								*<0.001*
1	17 (6.0)	0	2 (5.3)	12 (6.3)	11 (5.5)	3 (7.1)	3 (2.3)	11 (9.9)
2	37 (13.0)	1 (9.1)	1 (2.6)	25 (13.0)	24 (11.9)	4 (9.5)	7 (5.3)	21 (18.9)
3	193 (68.0)	8 (72.7)	26 (68.4)	135 (70.3)	143 (71.1)	27 (64.3)	104 (78.2)	67 (60.4)
4	37 (13.0)	2 (18.2)	9 (23.7)	20 (10.4)	23 (11.4)	8 (19.0)	19 (14.3)	12 (10.8)
Differentiation (%)								
Moderate	145 (64.2)	4 (44.4)	16 (64.0)	104 (65.4)	104 (64.2)	20 (64.5)	69 (64.5)	56 (64.4)
Poor	23 (10.2)	0 (0)	3 (12.0)	18 (11.3)	15 (9.3)	6 (19.4)	11 (10.3)	10 (11.5)
Good	58 (25.7)	5 (55.6)	6 (24.0)	37 (23.3)	43 (26.5)	5 (16.1)	27 (25.2)	21 (24.1)
Mucinous aspect(%)								
No	233 (83.5)	7 (63.6)	28 (77.8)	165 (86.8)	164 (83.2)	36 (85.7)	105 (80.2)	96 (88.1)
Fully	33(11.8)	4 (36.4)	6 (16.7)	15 (7.9)	24 (12.2)	3 (7.1)	18 (13.7)	9 (8.3)
Partly	13 (4.6)	0 (0)	2 (5.6)	10 (5.3)	9 (4.6)	3 (7.1)	8 (6.1)	4 (3.7)
Microsatellite stability(%)								
MSS	168 (84.8)	6 (66.7)	25 (86.2)	132 (87.4)	136 (86.6)	29 (85.3)	84 (81.6)	81 (92.0)
MSI	30 (15.2)	3 (33.3)	4 (13.8)	19 (12.6)	21 (13.4)	5 (14.7)	19 (18.4)	7 (8.0)

This table shows the patient characteristics of the entire colon cancer cohort (n=285) and stratified according to HLA class 1, HLA-EG and Foxp3+ staining. Only T stage was significantly related to Foxp3+. Abbreviations. MSS; microsatellite stability, MSI; Microsatellite instability.
Note: HLA-EG is a combination of HLA-E and HLA-G (explained in the material and methods section).

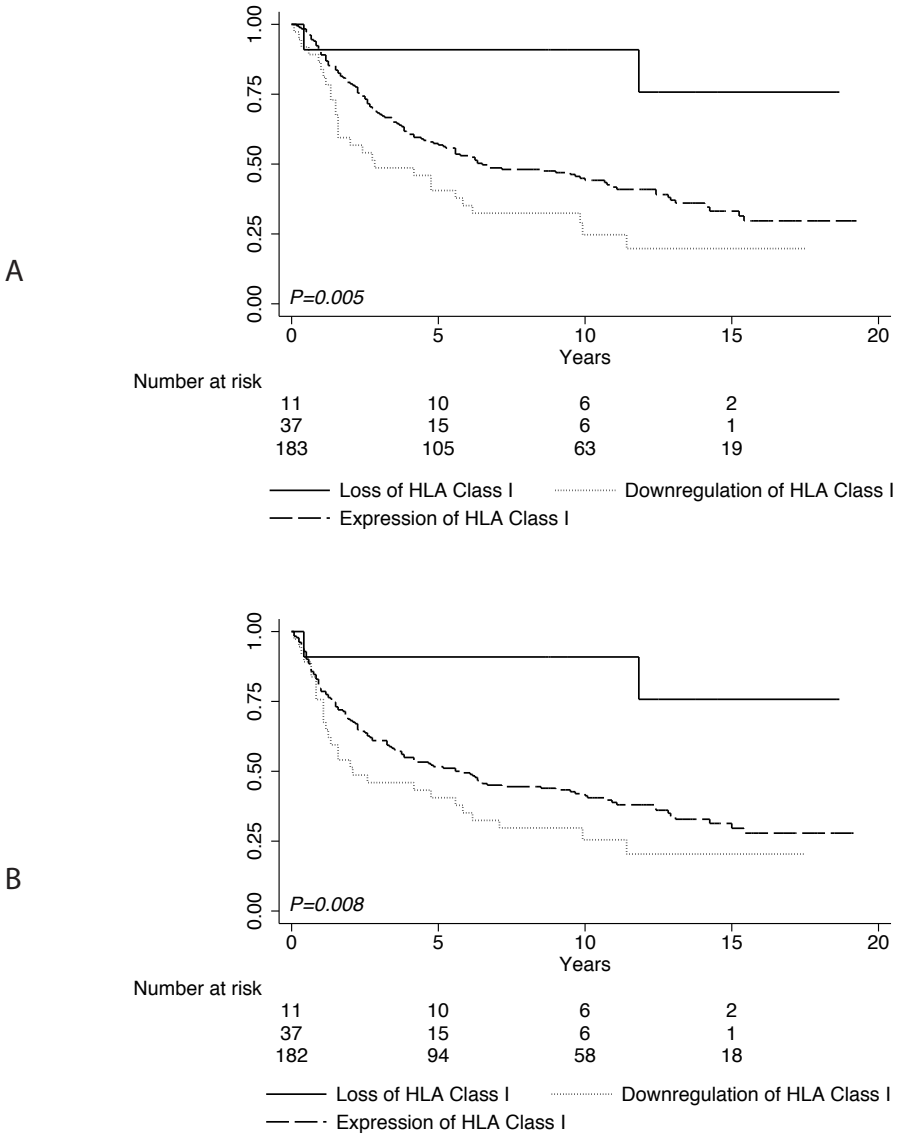


Figure 3: Survival curves stratified for HLA class I tumor expression in colon cancer.
A) Kaplan Meier curve for OS in the study population of 285 colon cancer patients stratified for HLA class I tumor expression status. B) Kaplan Meier curve for DFS in the study population of 285 colon cancer patients again stratified for HLA Class I tumor expression.

Foxp3+ cells

The number of Foxp3+ cells could be evaluated in 245 patients, because, due to staining artifacts and loss of material during the staining procedure, the IHC results of 40 cases could not be analyzed. The mean number of positive cells per tumor punch was 19 with a median of 12.0. In 4.1% (n=10) of the patients no Foxp3+ cells were present. Representative images of Foxp3+ staining are shown in Figure 1. Patients with expression of HLA class I showed borderline significantly higher levels of Foxp3+ cells in their tumor punches compared to HLA class I downregulation or loss: mean in expression

group 21 vs. a mean of 12 and 14 positive cells in the downregulation group and loss of HLA class I group respectively; p-value 0.07. Patients with stage 1 tumors showed significantly higher levels of Foxp3+ cells compared to patients with stage 2, stage 3 and stage 4 tumors: mean level of Foxp3+ cells in stage 1 tumors was 38 compared to 13, 17 and 20 for the stage 2, 3 and 4 tumors, *p*-value <0.001. For further analysis Foxp3+ was categorized as below vs. above median based on the median due to the skewness in the spread of the data. Frequencies are shown in Figure 2. The presence of Foxp3+ cells in the tumor micro-environment was not related to OS (logrank *p*-value 0.114) or DFS (logrank *p*-value 0.155).

HLA-E and HLA-G

Representative images for HLA-E and HLA-G and frequencies in the different groups are shown in Figure 1 and 2. HLA-E and HLA-G were not related to OS and DFS (logrank *p*-values for OS 0.809 and 0.239 respectively, logrank *p*-values for DFS 0.876 and 0.117 respectively). None of the clinico-pathological characteristics were significantly related to tumor expression of HLA-E or HLA-G (data not shown).

A combined variable of HLA-E and HLA-G scores was created (cEG). Expression was considered positive when both HLA-E and HLA-G were expressed (HLA-E+/-G+ further denoted as cEG+) and negative when either HLA-E or HLA-G was not expressed (HLA-E+/-G- or HLA-E-/-G+ or HLA-E-/-G- further denoted as cEG-). Positive cEG was found in 14.7% (42 of 244) of tumors. Patient characteristics and data on the combined variable HLA-E and -G expression can be found in Table I. None of the clinico-pathological variables shown in Table I were significantly related to tumor expression of cEG. cEG was not significantly related to OS (logrank *p*-value 0.245) and DFS (logrank *p*-value 0.100).

Multivariable analysis

Both for OS and DFS a univariate analysis was performed for the following parameters: sex, age, TNM stage, HLA class I expression status, mucinous differentiation, tumor grade, adjuvant therapy and microsatellite status. In the univariate analysis for OS, age (*p*-value <0.001), TNM status (*p*-value <0.001) and HLA class I expression status (*p*-value 0.011) were significant predictors of survival. The same was true for the univariate analysis for DFS with a *p*-value of <0.001 for age and TNM status and a *p*-value of 0.02 for HLA class I expression. Therefore all three were included in the multivariable analysis. In this analysis age and TNM stage remained significant for both OS and DFS (OS and DFS *p*-values all <0.001): HLA class I was a borderline independent significant predictor for OS (*p*-value 0.08) (Table IIa and IIb).

Table IIa: Univariate and multivariable analyses of Overall Survival (OS) in the different immune markers and in the tumor immune phenotypes

	Univariate analysis			Multivariable analysis*		
	HR	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
HLA class I			0.011			0.08
Loss	1.0			1.0		
Downregulation	7.3	1.7-30.8		4.3	1.0-18.5	
Expression	4.9	1.2-20.0		3.1	0.7-12.6	
Foxp3+			0.116			
Below median	1.0					
Above median	0.8	0.6-1.1				
HLA-E			0.810			
Negative	1.0					
Positive	1.0	0.7-1.4				
HLA-G			0.242			
Negative	1.0					
Positive	1.2	0.9-1.8				
HLA-EG			0.248			
Negative	1.0					
Positive	1.3	0.8-1.9				
Immune phenotypes			0.001			0.009
Phenotype a	1.0			1.0		
Phenotype b	4.7	1.2-19.0		2.9	0.7-11.9	
Phenotype c	8.2	2.0-34.2		4.8	1.1-20.2	

**Corrected for sex, age, TNM stage, HLA class I expression status, mucinous differentiation, tumor grade, adjuvant therapy and microsatellite status. Only significant variables in univariate analysis are corrected in multivariable analysis. Note: HLA-EG is a combination of HLA-E and HLA-G (as explained in the results section).*

Table IIb: Univariate and multivariable analyses of Disease Free Survival (DFS) in the different immune markers and in the tumor immune phenotypes.

	Univariate analysis			Multivariable analysis*		
	HR	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
HLA class I			0.021			0.104
Loss	1.00			1.00		
Downregulation	7.2	1.7-30.1		4.6	1.1-19.7	
Expression	5.4	1.3-21.8		3.7	0.9-15.0	
Foxp3+			0.159			
Below median	1.00					
Above median	0.8	0.6-1.1				

Table IIb: Univariate and multivariable analyses of Disease Free Survival (DFS) in the different immune markers and in the tumor immune phenotypes. *Continued*

	Univariate analysis			Multivariable analysis*		
	HR	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
HLA-E			0.877			
Negative	1.00					
Positive	0.97	0.7-1.4				
HLA-G			0.121			
Negative	1.00					
Positive	1.3	0.9-1.9				
HLA-EG			0.104			
Negative	1.00					
Positive	1.4	0.9-2.1				
Immune phenotypes			0.002			0.013
Phenotype a	1.00			1.00		
Phenotype b	5.1	1.3-20.7		3.5	0.8-14.2	
Phenotype c	8.4	2.0-34.9		5.4	1.3-22.7	

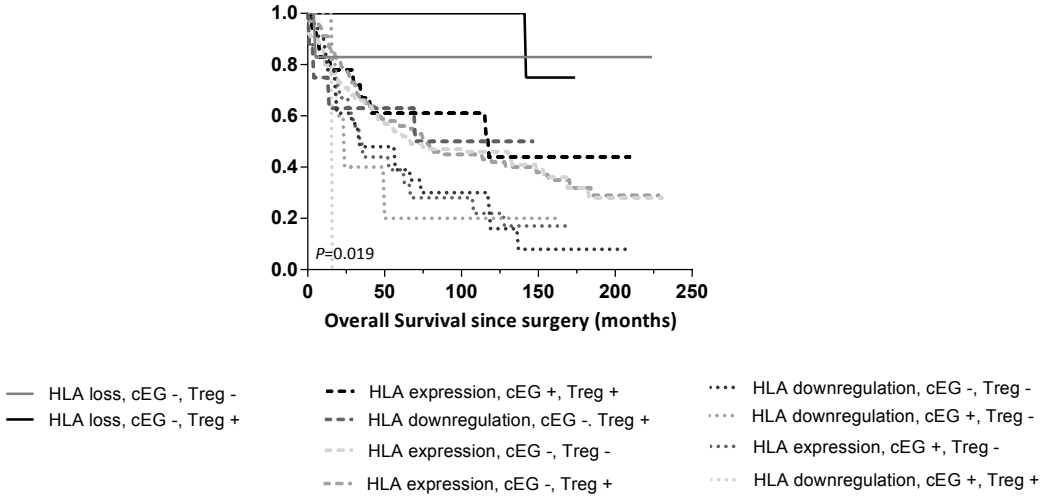
**Corrected for sex, age, TNM stage, HLA class I expression status, mucinous differentiation, tumor grade, adjuvant therapy and microsatellite status. Only significant variables in univariate analysis are corrected in multivariable analysis. Note: HLA-EG is a combination of HLA-E and HLA-G (as explained in the results section).*

Analysis of tumor immune phenotypes

Except for HLA class I, none of the tumor immune markers showed a significant correlation with patients' clinical outcome. The interaction between tumor cells and immune cells, however, is complex and multifaceted. Therefore, we hypothesized that analysis of combined tumor immune markers; describing a tumor's immune phenotype may better reflect outcome of the interaction between tumor cells and the immune system. We combined all of the data into one combined variable. The Kaplan Meier curves performed with this combined variable indeed revealed 3 distinct patterns in relation to patient outcome (Figure 4 and 5). The entire population could be divided in 3 phenotypes:

a) Strong immune system tumor recognition: Patients with tumors that showed loss of HLA class I expression, presence of Foxp3+ cells in the tumor micro-environment, and negative cEG expression (n=11).
b) Intermediate immune system tumor recognition: Patients with tumors that showed downregulation of HLA Class I expression and negative cEG expression, but were found to have Foxp3+ cells in the tumor micro-environment or patients with tumors that showed normal HLA class I expression irrespective of cEG expression and the presence of Foxp3+ cells (n=184).

A



B

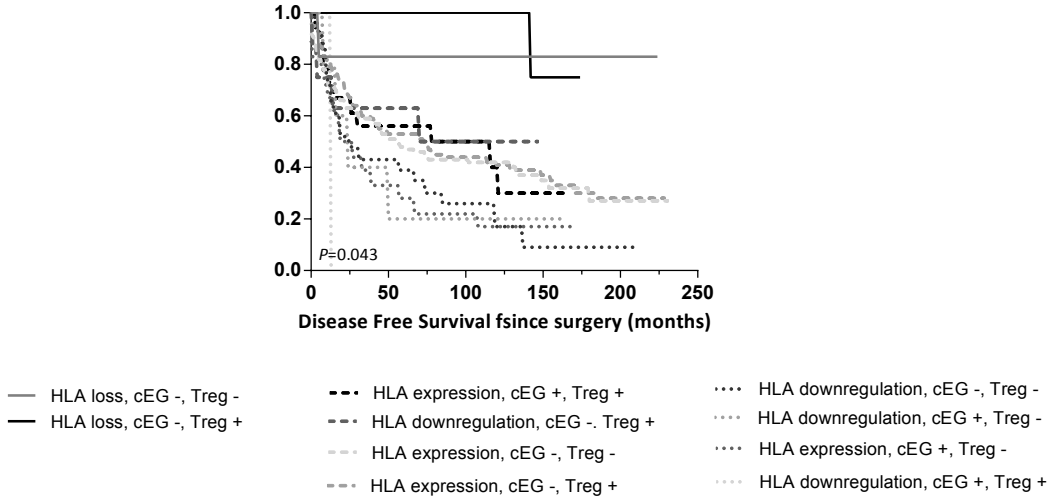
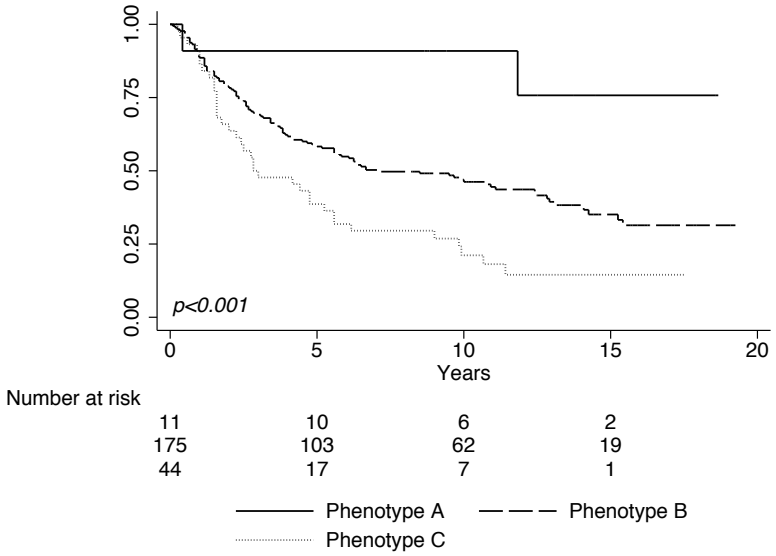


Figure 4: Survival curves stratified for combined tumor expression of HLA class I, HLA-E, HLA-G and Foxp3+ in colon cancer.

A) Kaplan Meier curve for OS in the study population of 285 colon cancer patients stratified for all the different combinations between tumor expression of HLA class I, combined expression of HLA-E and HLA-G (cEG) and the presence of Foxp3+ cells (Tregs) based on which 3 distinct patters could be distinguished, as shown in Figure 5. B) Kaplan Meier curve for DFS in the study population of 285 colon cancer patients stratified for all the different combinations between tumor expression of HLA class I, combined expression of HLA-E and HLA-G (cEG) and the presence of Foxp3+ cells (Tregs) based on which 3 distinct patterns could be distinguished, as shown in Figure 5.

A



B

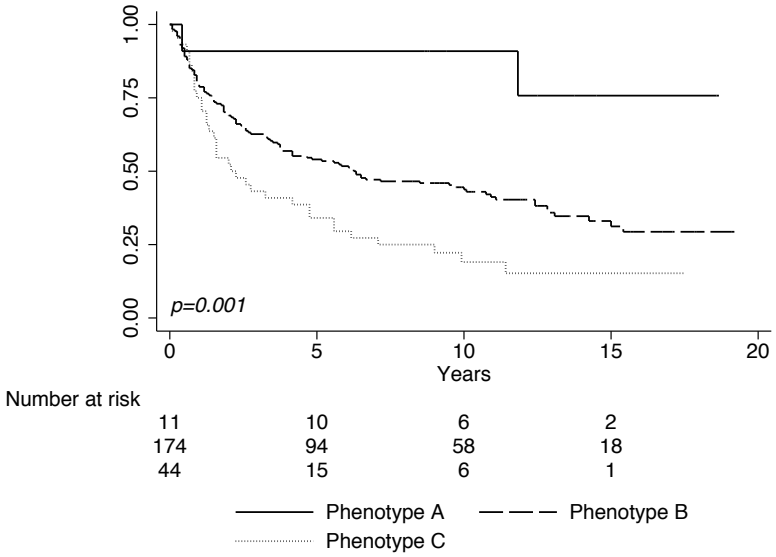


Figure 5: Survival curves stratified for immune phenotypes in colon cancer.
A) Kaplan Meier curve for OS in the study population of 285 colon cancer patients stratified for all the different combinations between tumor expression of HLA class I, combined expression of HLA-E and HLA-G (cEG) and the presence of Foxp3+ cells (Tregs) based on which 3 immune phenotypes could be distinguished. See *Results* section for explanation of the phenotypes. B) Kaplan Meier curve for DFS in the study population of 285 colon cancer patients stratified for all the different combinations between tumor expression of HLA class I, combined expression of HLA-E and HLA-G (cEG) and the presence of Foxp3+ cells (Tregs) based on which 3 immune phenotypes could be distinguished. See *Results* section for explanation of the phenotypes.

c) Poor immune system tumor recognition: Patients with tumors showing normal or downregulated HLA class I and no presence of Foxp3+ cells irrespective of their cEG expression (n=460).

These three phenotypes showed significant differences for OS (logrank *p*-value <0.001) and DFS (logrank *p*-value 0.001). The HRs of the three phenotypes for OS and DFS are shown in Table IIa and IIb.

Multivariable analysis

Again, both for OS and DFS a univariate analysis was performed for the following parameters: sex, age, TNM stage, tumor immune phenotype, mucinous differentiation, tumor grade, adjuvant therapy, and microsatellite status. In univariate analysis, next to age and TNM status, the tumor immune phenotype was a significant predictor for OS (*p*-value 0.001) and DFS (*p*-value 0.002). Therefore all three these parameters were included in multivariable analysis. The tumor immune phenotype was an independent significant predictor for both OS (*p*-value 0.009) and DFS (*p*-value 0.013) and HRs are shown in table IIa and IIb.

DISCUSSION

Tumor-immune interactions may be important for the prognosis of cancer patients [17]. In this study, we showed that by combining the immune-related markers HLA class I, HLA-E, HLA-G and Foxp3+, we were able to determine three distinct patterns in survival, which might represent how immune surveillance controls tumor growth and metastasis.

The first marker of tumor-immunogenicity used was the level of HLA class I expression of cancer cells. Our results are comparable with the results of other studies that were able to determine a prognostic effect of the HLA class I status in colon cancer [35;39]. Watson *et al.* showed that tumors with downregulation of HLA class I had a worse survival comparable with our results [39]. In contrast, Menon *et al.* showed a survival benefit in patients with downregulated HLA-A tumors [35]. However, when HLA-A and HLA-B/C were combined, statistical significance was lost. Furthermore, patients with expression of HLA class I were related to a better survival in the study by Watson *et al.,* whereas our study showed an improved survival in patients with loss of HLA class I expression. Possible explanations for these differences might be a different definition for HLA class I expression, differences in staining techniques and scoring or a different patient cohort, especially regarding the number of tumors showing microsatellite instability (MSI), which is associated with loss of HLA class I and a better prognosis [56;57]. In our study, 33% of the tumors with loss of HLA class I showed the MSI phenotype, in comparison to 14% and 13% for HLA class I downregulation and expression. Results from Menon *et al.* showed that 50%

of the tumors with loss of HLA class I had the MSI phenotype. Unfortunately, *Watson et al.* did not mention microsatellite status of their study cohort.

As hypothesized, loss of HLA class I expression in tumor cells could also be related to a better patient survival because such cells, once they metastasize to the bloodstream, are eliminated by NK cell attacks [35;39;58]. Tumors with loss of HLA class I have also shown to have significantly higher NK cell infiltration [15]. More interestingly, the tumors showing loss of HLA class I in our cohort were also the ones that showed to be negative for HLA-E and -G expression (phenotype a). Absence of the HLA-E and -G expression makes them even more susceptible to NK cell elimination [17-20]. Furthermore, this is also confirmed by CRC tumors with loss of HLA class I expression who do not metastasize to the liver [59].

The presence of the third marker Foxp3+ is thought to represent the inhibition of host-protective antitumor responses. When stimulated, they inhibit the function of CTL [6]. Although the exact mechanism by which these cells are drawn into the tumor micro-environment remains unexplained, their immunosuppressive effect has been proven with a high density of tumor-infiltrating Foxp3+ cells found to be associated with an unfavorable prognosis in a wide range of human carcinomas, including breast and lung cancer [60;61]. However, in colon cancer different results are reported as well [37;38]. One possible explanation for these opposite results might be a different micro-environment of colon cancer, which is colonized with many gastro-intestinal bacteria, triggering the production of pro-inflammatory cytokines causing tumor-enhancing effects. Instead of the specificity of infiltrating T-cells for tumor-antigens, T-cells in colon cancer could be more specific for the microflora and suppress inflammation and immune responses from bacterial invasion, resulting in an anti-tumorigenic effect, which could explain the better prognosis of patients with tumors with a strong Foxp3+ infiltration [62]. We were not able to demonstrate differences in disease outcome for Foxp3+ tumor infiltration supporting this latter hypothesis, but we did see differences in Foxp3+ infiltration if we combined them with HLA class I expression and with HLA-E and -G expression, especially in patients who have retained their HLA class I expression. Patients with normal HLA class I expression and absence of Foxp3+ cell infiltration showed a worse patient outcome. We hypothesize that the tumors of these patients have had a minimal CTL attack because the HLA class I expression is preserved, indicating no selective outgrowth of HLA class I-negative or downregulated tumors directed by CTL. Since CTL and Foxp3+ cells show opposing actions [27] and CTLs are supposed to be absent in these tumors, Foxp3+ cell infiltration might not be necessary. These tumors could therefore progress aggressively as immune surveillance is poor. In contrary, tumors with HLA class I expression, which were able to attract Foxp3+ cells, showed a slightly better prognosis. In this case, Foxp3+ cell infiltration might indicate CTL activity resulting in suppression of tumor growth.

Therefore, in our opinion, the clinical relevance of the studies by Watson *et al.* and several others does not provide an optimal perspective on prognosis [35;39], because ex-

pression of a single immune marker is not sufficient for the selection of high-risk colon cancer patients or treatment allocation. As shown by our results and previous studies, immune markers are related to each other [29;46;63;64].

When all markers were combined, patients showing the worst prognosis were patients with HLA class I downregulation, negative or positive cEG expression and absence of Foxp3+ cells denoted as phenotype c. We hypothesize that these poor immune system recognized tumors were able to elicit only a minimal CTL attack because they partly preserved HLA class I expression and subsequently attracted little to no Foxp3+ cells in their tumor micro-environment. Furthermore, these tumors showed a positive expression of HLA-E and -G, further escaping immune surveillance through inhibition of NK cell recognition [17-20]. These tumor cells can therefore quickly progress to the bloodstream and might eventually metastasize.

It is important to realize that what we are evaluating is just a 'snapshot' of the ongoing process of cancer immuno-editing in the patient's primary tumor at time of resection. Still, from a clinical point of view, at the patient's bedside this is usually the only data available based on which clinical decision making has to take place and these data can actually be of clinical value to, for example, the allocation of adjuvant therapy as opposed by De Kruijf *et al.* in breast cancer and other studies [29;65;66].

Our study does have a few limitations. Not all combinations between HLA class I, HLA-E and -G and Foxp3+ were present in our cohort. There was no representation of tumors with loss of HLA class I, which were HLA-E and -G positive. Therefore we were not able to investigate the prognosis of these tumors, but we hypothesize that these tumors have a worse prognosis as these tumors might escape NK cell attack. Although there is a physiological correlation between HLA-E and HLA class I molecules, this has been found to be disturbed in tumors, suggesting further escape from immune recognition through upregulation of HLA-E [21;46]. To truly investigate these tumors, our study has to be validated in a bigger cohort. Second, the antibodies we used for HLA class I detection only detected the heavy chain, but not the trimeric complex consisting of β2-microglobuline heavy chain and antigen [67]. Therefore we should be careful using the term total loss of HLA class I. Third, we did not investigated the role of NK cells in patients with loss or downregulation of HLA class I, possibly explaining the positive prognostic effect of patients with loss of HLA class I expression. However, NK cell infiltration at the tumor site is scarce, indicating that tumor staining for NK cells might be minimally informative [40].

In conclusion we were able to identify local immune escape mechanisms of colon cancer, where the presence of Foxp3+ cell infiltration favors a better prognosis, indicating CTL activity. HLA-E and -G expression might play a pivotal role in distant immune escape mechanisms, where in case of loss or downregulation of HLA class I, HLA-E and -G expression determines distant metastases and prognosis of colon cancer patients. Furthermore we were able to determine three distinct survival patterns in colon cancer

patients based on immune surveillance. In the future these findings might contribute to better treatment allocation and maybe even the development of new cancer immunotherapies.

REFERENCE LIST

(1) Halvorsen TB, Seim E. Association between invasiveness, inflammatory reaction, desmoplasia and survival in colorectal cancer. *J Clin Pathol* 1989;42:162-166.

(2) Murphy J, O'Sullivan GC, Lee G et al. The inflammatory response within Dukes' B colorectal cancers: implications for progression of micrometastases and patient survival. *Am J Gastroenterol* 2000;95:3607-3614.

(3) Menon AG, Janssen-van Rhijn CM, Morreau H et al. Immune system and prognosis in colorectal cancer: a detailed immunohistochemical analysis. *Lab Invest* 2004;84:493-501.

(4) Naito Y, Saito K, Shiiba K et al. CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. *Cancer Res* 1998;58:3491-3494.

(5) Cavallo F, De GC, Nanni P, Forni G, Lollini PL. 2011: the immune hallmarks of cancer. *Cancer Immunol Immunother* 2011;60:319-326.

(6) Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 2011;331:1565-1570.

(7) Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* 2002;3:991-998.

(8) Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* 2004;21:137-148.

(9) Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. *Annu Rev Immunol* 2004;22: 329-360.

(10) Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol* 2006;6:836-848.

(11) Smyth MJ, Dunn GP, Schreiber RD. Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. *Adv Immunol* 2006;90:1-50.

(12) Swann JB, Smyth MJ. Immune surveillance of tumors. *J Clin Invest* 2007;117:1137-1146.

(13) Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ. Natural innate and adaptive immunity to cancer. *Annu Rev Immunol* 2011;29:235-271.

(14) Halvorsen TB, Seim E. Association between invasiveness, inflammatory reaction, desmoplasia and survival in colorectal cancer. *J Clin Pathol* 1989;42:162-166.

(15) Menon AG, Janssen-van Rhijn CM, Morreau H et al. Immune system and prognosis in colorectal cancer: a detailed immunohistochemical analysis. *Lab Invest* 2004;84:493-501.

(16) Murphy J, O'Sullivan GC, Lee G et al. The inflammatory response within Dukes' B colorectal cancers: implications for progression of micrometastases and patient survival. *Am J Gastroenterol* 2000;95:3607-3614.

(17) Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nat Immunol* 2002;3:999-1005.

(18) Marin R, Ruiz-Cabello F, Pedrinaci S et al. Analysis of HLA-E expression in human tumors. *Immunogenetics* 2003;54:767-775.

(19) Wischhusen J, Waschbisch A, Wiendl H. Immune-refractory cancers and their little helpers—an extended role for immunetolerogenic MHC molecules HLA-G and HLA-E? *Semin Cancer Biol* 2007; 17:459-468.

(20) Zilberman S, Schenowitz C, Agaugue S et al. HLA-G1 and HLA-G5 active dimers are present in malignant cells and effusions: the influence of the tumor microenvironment. *Eur J Immunol* 2012; 42:1599-1608.

(21) Palmisano GL, Contardi E, Morabito A, Gargaglione V, Ferrara GB, Pistillo MP. HLA-E surface expression is independent of the availability of HLA class I signal sequence-derived peptides in human tumor cell lines. *Hum Immunol* 2005;66:1-12.

(22) Nosho K, Baba Y, Tanaka N et al. Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer, and prognosis: cohort study and literature review. *J Pathol* 2010;222:350-366.

(23) Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat Rev Immunol* 2006;6:715-727.

(24) Curiel TJ. Tregs and rethinking cancer immunotherapy. *J Clin Invest* 2007;117:1167-1174.

(25) Needham DJ, Lee JX, Beilharz MW. Intra-tumoural regulatory T cells: a potential new target in cancer immunotherapy. *Biochem Biophys Res Commun* 2006;343:684-691.

(26) Zou W. Regulatory T cells, tumour immunity and immunotherapy. *Nat Rev Immunol* 2006;6:295-307.

(27) Liu F, Lang R, Zhao J et al. CD8(+) cytotoxic T cell and FOXP3(+) regulatory T cell infiltration in relation to breast cancer survival and molecular subtypes. *Breast Cancer Res Treat* 2011;130:645-655.

(28) Curiel TJ, Coukos G, Zou L et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004;10:942-949.

(29) de Kruijf EM, van Nes JG, Sajet A et al. The predictive value of HLA class I tumor cell expression and presence of intratumoral Tregs for chemotherapy in patients with early breast cancer. *Clin Cancer Res* 2010;16:1272-1280.

(30) Gao Q, Qiu SJ, Fan J et al. Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. *J Clin Oncol* 2007;25:2586-2593.

(31) Hiraoka N, Onozato K, Kosuge T, Hirohashi S. Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. *Clin Cancer Res* 2006;12:5423-5434.

(32) Benevolo M, Mottolese M, Piperno G et al. HLA-A, -B, -C expression in colon carcinoma mimics that of the normal colonic mucosa and is prognostically relevant. *Am J Surg Pathol* 2007;31:76-84.

(33) Deng L, Zhang H, Luan Y et al. Accumulation of foxp3+ T regulatory cells in draining lymph nodes correlates with disease progression and immune suppression in colorectal cancer patients. *Clin Cancer Res* 2010;16:4105-4112.

(34) Loddenkemper C, Schernus M, Noutsias M, Stein H, Thiel E, Nagorsen D. In situ analysis of FOXP3+ regulatory T cells in human colorectal cancer. *J Transl Med* 2006;4:52.

(35) Menon AG, Morreau H, Tollenaar RA et al. Down-regulation of HLA-A expression correlates with a better prognosis in colorectal cancer patients. *Lab Invest* 2002;82:1725-1733.

(36) Moller P, Momburg F, Koretz K et al. Influence of major histocompatibility complex class I and II antigens on survival in colorectal carcinoma. *Cancer Res* 1991;51:729-736.

(37) Salama P, Phillips M, Grieu F et al. Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol* 2009;27:186-192.

(38) Suzuki H, Chikazawa N, Tasaka T et al. Intratumoral CD8(+) T/FOXP3 (+) cell ratio is a predictive marker for survival in patients with colorectal cancer. *Cancer Immunol Immunother* 2010;59:653-661.

(39) Watson NF, Ramage JM, Madjd Z et al. Immunosurveillance is active in colorectal cancer as down-regulation but not complete loss of MHC class I expression correlates with a poor prognosis. *Int J Cancer* 2006;118:6-10.

(40) Sandel MH, Speetjens FM, Menon AG et al. Natural killer cells infiltrating colorectal cancer and MHC class I expression. *Mol Immunol* 2005;42:541-546.

(41) Zeestraten EC, Van Hoesel AQ, Speetjens FM et al. FoxP3- and CD8-positive Infiltrating Immune Cells Together Determine Clinical Outcome in Colorectal Cancer. *Cancer Microenviron* 2011.

(42) Ye SR, Yang H, Li K, Dong DD, Lin XM, Yie SM. Human leukocyte antigen G expression: as a significant prognostic indicator for patients with colorectal cancer. *Mod Pathol* 2007;20:375-383.

(43) Yie SM, Yang H, Ye SR, Li K, Dong DD, Lin XM. Expression of human leukocyte antigen G (HLA-G) correlates with poor prognosis in gastric carcinoma. *Ann Surg Oncol* 2007;14:2721-2729.

(44) Yie SM, Yang H, Ye SR, Li K, Dong DD, Lin XM. Expression of HLA-G is associated with prognosis in esophageal squamous cell carcinoma. *Am J Clin Pathol* 2007;128:1002-1009.

(45) Yie SM, Yang H, Ye SR, Li K, Dong DD, Lin XM. Expression of human leucocyte antigen G (HLA-G) is associated with prognosis in non-small cell lung cancer. *Lung Cancer* 2007;58:267-274.

(46) de Kruijf EM, Sajet A, van Nes JG et al. HLA-E and HLA-G expression in classical HLA class I-negative tumors is of prognostic value for clinical outcome of early breast cancer patients. *J Immunol* 2010; 185:7452-7459.

(47) Schiffmann L, Eiken AK, Gock M, Klar E. Is the lymph node ratio superior to the Union for International Cancer Control (UICC) TNM system in prognosis of colon cancer? *World J Surg Oncol* 2013; 11:79.

(48) Generali D, Bates G, Berruti A et al. Immunomodulation of FOXP3+ regulatory T cells by the aromatase inhibitor letrozole in breast cancer patients. *Clin Cancer Res* 2009;15:1046-1051.

(49) Hill JA, Feuerer M, Tash K et al. Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity* 2007;27:786-800.

(50) Lo ME, Sibilio L, Melucci E et al. HLA-E: strong association with beta2-microglobulin and surface expression in the absence of HLA class I signal sequence-derived peptides. *J Immunol* 2008;181: 5442-5450.

(51) Menier C, Saez B, Horejsi V et al. Characterization of monoclonal antibodies recognizing HLA-G or HLA-E: new tools to analyze the expression of nonclassical HLA class I molecules. *Hum Immunol* 2003;64:315-326.

(52) Polakova K, Kuba D, Russ G. The 4H84 monoclonal antibody detecting beta2m free nonclassical HLA-G molecules also binds to free heavy chains of classical HLA class I antigens present on activated lymphocytes. *Hum Immunol* 2004;65:157-162.

(53) Zhao L, Teklemariam T, Hantash BM. Reassessment of HLA-G isoform specificity of MEM-G/9 and 4H84 monoclonal antibodies. *Tissue Antigens* 2012;80:231-238.

(54) Chew SF, Kanaan C, Tait BD. HLA expression and cancer—14th IHIWS immunohistochemistry quality control exercise exchange results. *Tissue Antigens* 2007;69 Suppl 1:248-251.

(55) Zeestraten EC, Maak M, Shibayama M et al. Specific activity of cyclin-dependent kinase I is a new potential predictor of tumour recurrence in stage II colon cancer. *Br J Cancer* 2012;106:133-140.

(56) Dierssen JW, de Miranda NF, Ferrone S et al. HNPCC versus sporadic microsatellite-unstable colon cancers follow different routes toward loss of HLA class I expression. *BMC Cancer* 2007;7:33.

(57) Mouradov D, Domingo E, Gibbs P et al. Survival in stage II/III colorectal cancer is independently predicted by chromosomal and microsatellite instability, but not by specific driver mutations. *Am J Gastroenterol* 2013.

(58) Hokland M, Kuppen PJ. Natural killer cells: from "disturbing" background to central players of immune responses. *Mol Immunol* 2005;42:381-383.

(59) Tikidzhieva A, Benner A, Michel S et al. Microsatellite instability and Beta2-Microglobulin mutations as prognostic markers in colon cancer: results of the FOGT-4 trial. *Br J Cancer* 2012;106: 1239-1245.

(60) Bates GJ, Fox SB, Han C et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol* 2006;24:5373-5380.

(61) Petersen RP, Campa MJ, Sperlazza J et al. Tumor infiltrating Foxp3+ regulatory T-cells are associated with recurrence in pathologic stage I NSCLC patients. *Cancer* 2006;107:2866-2872.

(62) Ladoire S, Martin F, Ghiringhelli F. Prognostic role of FOXP3+ regulatory T cells infiltrating human carcinomas: the paradox of colorectal cancer. *Cancer Immunol Immunother* 2011;60:909-918.

(63) Angell H, Galon J. From the immune contexture to the Immunoscore: the role of prognostic and predictive immune markers in cancer. *Curr Opin Immunol* 2013;25:261-267.

(64) Galon J, Costes A, Sanchez-Cabo F et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006;313:1960-1964.

(65) Ghiringhelli F, Larmonier N, Schmitt E et al. CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur J Immunol* 2004;34:336-344.

(66) Ladoire S, Arnould L, Apetoh L et al. Pathologic complete response to neoadjuvant chemotherapy of breast carcinoma is associated with the disappearance of tumor-infiltrating foxp3+ regulatory T cells. *Clin Cancer Res* 2008;14:2413-2420.

(67) Perosa F, Luccarelli G, Prete M, Favoino E, Ferrone S, Dammacco F. Beta 2-microglobulin-free HLA class I heavy chain epitope mimicry by monoclonal antibody HC-10-specific peptide. *J Immunol* 2003;171:1918-1926.

CHAPTER 3

Prognostic value of HLA class I, HLA-E, HLA-G and Tregs in rectal cancer: a retrospective cohort study

Marlies S. Reimers, Charla C. Engels, Hans Morreau, Gerrit Jan Liefers, Cornelis J.H. van de Velde, Peter J.K. Kuppen

BMC Cancer, 2014 Jul 5; 14:486

ABSTRACT

Background

Evasion of immune surveillance and suppression of the immune system are important hallmarks of tumorigenesis. The goal of this study was to establish distinct patterns that reflect a rectal tumors' immune-phenotype and to determine their relation to patient outcome.

Methods

The study population consisted of 495 Stage I-IV non-preoperatively treated rectal cancer patients of which a tissue micro array (TMA) was available. Sections of this TMA were immunohistochemically stained and quantified for presence of Foxp3+ cells (Tregs) and tumor expression of HLA Class I and non-classical HLA-E and HLA-G. All markers were, separate and combined, analyzed for clinical prognostic value.

Results

Expression of HLA class I (DFS HR 0.637 (0.458-0.886), p=0.013), Foxp3+ infiltration above median (OS HR 0.637 (0.500-0.813), $p<0.001$ and DFS HR 0.624 (0.491-0.793), $p<0.001$) and expression of HLA-G (DFS HR 0.753 (0.574-0.989), $p=0.042$) were related to a better clinical prognosis. When these markers were combined, patients with 2 or 3 markers associated with poor prognosis (loss of HLA Class I, Foxp3+ below median, and weak HLA-G expression), showed a significantly worse survival (OS and DFS $p<0.001$). This immune-phenotype was an independent predictor for DFS (HR 1.56 (1.14-2.14), $p=0.019$).

Conclusions

In conclusion, rectal tumors showing loss of HLA class I expression, Foxp3+ infiltration below median and weak HLA-G expression were related to a worse OS and DFS. Combining these immune markers lead to the creation of tumor immune-phenotypes , which related to patient outcome and were significant independent clinical prognostic markers in rectal cancer.

BACKGROUND

The immune system has proven to play an important role in tumorigenesis and gained a lot of attention in cancer research [1-4]. Consequently, evasion of immune surveillance has become one of the important hallmarks of cancer [5]. Tumors are thought to be 'edited' through a Darwinian selection process into poorly immunogenic tumor variants, invisible to the immune system and able to grow progressively. Immuno-editing might influence patient's prognosis substantially [6].

We have described a few mechanisms responsible for evasion of immune surveillance below.

First, cytotoxic T-cells (CTL) are capable of destroying tumor cells by recognizing tumor-associated antigens (TAA) on the tumor cell surface presented by classical human leukocyte antigen (HLA) class I. Tumor cells can escape this CTL recognition through downregulation or complete loss of HLA class I, resulting in minimization of TAA expression and absence of CTL destruction [7-9]. Second, non-classical HLA-E and HLA-G also play an important role in immune surveillance. Presence of HLA-E and HLA-G causes an inhibitory signal to natural killer (NK) cells, resulting in further immune escape [7;10-14]. HLA-E is regularly expressed in various healthy tissues and correlates with HLA class I expression [15]. HLA-G is rarely found in healthy tissues, but is frequently observed in tumors [16]. Third, immune reactivity can become suppressed by the attraction of immunosuppressive regulatory T cells (Tregs) into the tumor microenvironment [17;18].

In colorectal cancer (CRC), the presence of Tregs in the tumor micro-environment has been related to a worse prognosis in some studies, although other studies showed an inverse association [19-22]. Loss of HLA Class I tumor expression was related to a better prognosis in CRC in most studies [14;23] and HLA-E and HLA-G tumor expression has been correlated with a poor prognosis and tumor progression [24;25].

In rectal cancer specifically, only a few studies reported on the role of the immune system, in which expression of HLA Class I was related to a better prognosis [26;27]. Recently, more studies showed differences in biology between colon- and rectal cancer [28-30]. Unfortunately, most studies so far have focused on CRC and did not perform separate analyses. Furthermore, often only one immune marker was investigated in CRC, while recent studies showed the complex interaction between the different mechanisms of immune-escape [6;31;32].

In this study we therefore aimed to investigate the immune-related biomarkers HLA Class I, HLA-E and -G and Tregs, determined with immunohistochemistry, in rectal cancer specifically, and to establish distinct patterns that reflect immune-escape mechanisms of rectal cancer by combining these markers and relate these patterns to clinical outcome.

METHODS

Study population

The study cohort consisted of patients obtained from the non-preoperative treated arm of the Dutch TME trial (January 12th, 1996, DUT-KWF-CKVO-9504, EORTC-40971, EU-96020), a multicenter trial that evaluated total mesorectal excision (TME) surgery with or without preoperative radiotherapy (5 × 5 Gray) from 1996-1999 [33]. Radiotherapeutical, surgical and pathological procedures were standardized and quality-controlled [34]. Before the start of the TME trial the Medical Ethical Committee of the Leiden University Medical Center approved the trial and retrospective use of samples. Written informed consent for participation and retrospective use of samples was obtained from all patients enrolled in the TME trial. Previously, a tissue microarray (TMA) including 1208 patients (irradiated and non-irradiated) of the Dutch TME trial was available. Because of insufficient tissue on this TMA a new TMA was constructed for this study. Sufficient formalin-fixed paraffin-embedded tumor material was available for 495 non-preoperative radiotherapy-treated stage I-IV Dutch patients, resulting in a total study cohort of 495 rectal cancer patients who only had surgery.

Antibodies

The mouse monoclonal antibodies HCA2 and HC10 were used, which recognize the heavy chains of HLA Class I, these were kindly provided by Prof. Dr. J. Neefjes (NKI, Amsterdam, The Netherlands). The reactivity spectrum of HCA2 comprises all HLA-A chains (except HLA-A24), as well as some HLA-B, HLA-C, HLA-E, HLA-F, and HLA-G chains. HC10 reacts with HLA-B and HLA-C heavy chains and some HLA-A chains (HLA-A10, HLA-A28, HLA-A29, HLA-A30, HLA-A31, HLA-A32, HLA-A33) [31]. The mouse antibody against human Foxp3 (ab20034 clone 236A/E7; Abcam) was used for Treg identification. The reactivity spectrum of Foxp3 is composed of regulatory T cells and may include small numbers of CD8+ cells but is generally considered to be the best single marker for Treg identification [35;36]. For HLA-E and HLA-G identification mouse monoclonal antibodies against HLA-E (ab2216 clone MEM-E/02: AbCam) and HLA-G (4H84: Exbio, Czech Republic) were used [32]. MEM-E/02 recognizes denatured HLA-E [37;38], while 4H84 recognizes denatured HLA-G molecules and also binds to free heavy chains of classical HLA class I molecules [38-40].

TMA production and immunohistochemistry

Histo-pathological characteristics of tumor material from all patients were standardized and quality-controlled [33;34]. Sections from formalin-fixed paraffin-embedded (FFPE) tumor blocks of the primary tumors were cut for haematoxylin and eosin staining. Based on these slides, histopathologically representative tumor regions were identified and punched for preparation of tumor tissue microarray (TMA) blocks. From each donor

block, three 1.0 mm diameter tissue cores were punched from three different identified tumor areas to account for tumor heterogeneity and transferred into a receiver paraffin block using the TMA master (3DHISTECH, Budapest, Hungary). Immunohistochemical staining (IHC) for Foxp3+ cells, non-classical HLA-E and HLA-G, and classical HLA class I tumor expression was performed on 4 µm sections, which were cut from each receiver block and mounted on glass. For each type of primary antibodies, all slides were stained simultaneously to avoid inter-assay variation.

The sections were deparaffinized and rehydrated in accordance with standard protocol. Endogenous peroxidase was blocked for 20 minutes in 0.3% hydrogen peroxide in PBS. For antigen retrieval, slides for staining with HLA-E, HLA-G or Foxp3+ were boiled in a 0.01 M EDTA buffer (pH 8) for 10 minutes at maximum power in a microwave oven. Slides for staining with HCA2 and HC10 were boiled in a 0.1 M citrate buffer (pH 6). Sections were incubated overnight with Foxp3, HLA-E, or HLA-G antibodies at predetermined optimal dilution. The next day, after 30 minutes of incubation with Envision anti-mouse (K4001; DAKO Cytomation, Glostrup, Denmark), sections were visualized using diaminobenzidine solution (DAB). Tissue sections were counterstained with haematoxylin, dehydrated and finally mounted in pertex.

For the HCA2 and HC10 stainings a double staining was performed to better discriminate between stroma (using a mixture of anti-extracellular matrix antibodies that resulted in brown staining of tumor stroma) and tumor tissue (using a blue staining for the HLA expression to be determined) in the tissue sections. Sections were incubated overnight at room temperature with all primary antibodies simultaneously (anti-collagen I, anti- collagen VI, anti-elastin (all polyclonal rabbit antibodies obtained from AbCam) and HCA2 and HC10). Afterwards, sections were washed three times for 5 minutes in PBS and incubated for 30 minutes with Envision+ System HRP anti Rabbit (DAKO, Glostrup, Denmark). After washing the sections three times with PBS, sections were developed using Liquid DAB+ Substrate Chromogen System (DAKO, Glostrup, Denmark) following manufacturer's instructions for visualization of stromal tissue. Then, sections were washed again three times for 5 minutes in PBS followed by 30 minutes incubation with rabbit-anti-mouse antibodies (DAKO, Glostrup, Denmark). Afterwards , the sections were incubated with APAAP (DAKO, Glostrup, Denmark) diluted in PBS/BSA 1% for 30 minutes. And finally, sections were washed three times for 5 minutes in PBS followed by 20 minutes incubation with Vector-Blue following manufacturer's instructions for visualization of the HCA2 and HC10 antibodies, and mounted in Aquamount (Merck, Darmstadt, Germany).

For each patient, normal epithelium, stromal cells, or lymphoid cells served as internal positive control for HLA class I and HLA-E antibody reactivity [24]. Tonsil tissue served as external positive control for the HCA2 and HC10 stainings and placenta tissue slides for the HLA-E and HLA-G stainings. Slides from human tonsil tissue served as positive control

for Foxp3 staining. Tissue slides that underwent the whole immuno-histochemical staining without primary antibodies served as negative controls (Supplemental Figure 1).

Evaluation of immunohistochemistry

Microscopic analysis of HCA2, HC10, HLA-E and HLA-G expression and presence of Foxp3+ cells was performed by two independent observers in a blinded manner (M.S.R.: 100% of the cohort, C.C.E. 30% of the cohort). The kappa values for inter-observer agreement were all between 0.5 and 0.7, indicating substantial agreement between the two observers [41]. The scores of the three 1.0 mm punches were averaged. For HCA2 and HC10 the percentage of tumor cells with membranous staining was assessed. HLA class I expression status was determined according to the International HLA and Immunogenetics Workshop [42], with tumor cell HLA class I expression status defined as follows: loss of HLA class I expression: less than 5% of tumor cells expressing both HCA2 and HC10, downregulation of HLA class I; less than 5% of tumor cells expressing either of the markers, and expression of HLA class I: 5% or more expressing both markers. For HLA-E and HLA-G, intensity of tumor staining (absent (undetectable or faint in <20% of the cells), weak (faint to weak in 20% but ≤70% of the cells), moderate (weak to moderate in >70% of the cells) or strong intensity (intense in 20-70% of the cells)) was determined, based on previous studies [43;44]. The scores of the three 1.0 mm punches were averaged as well. For analysis these scores were further categorized as weak (absent and weak intensity together) versus strong (moderate and strong intensity together) tumor staining. Quantification of the number of Foxp3+ cells was microscopically assessed in the entire tumor punches of the TMA and the absolute number of positive cells was used for analysis, with the use of the median as cut-off value for categorization in two categories: Foxp3+ below median and Foxp3+ above median.

Statistical Analysis

Statistical analyses were performed using the statistical package SPSS (version 17.0 for Windows; SPSS Inc.). The Student's T-test and the Chi-squared test were used to evaluate associations between tumor expression of classical HLA Class I, non-classical HLA-E and HLA-G and tumor infiltration of Foxp3+ cells and various clinico-pathological variables. Overall Survival (OS) was defined as time of surgery until death; Disease Free Survival (DFS) as time of surgery until death or relapse of disease, whichever came first. The Kaplan-Meier method was used for calculation of survival probabilities and the Log-rank test for comparison of survival curves between expression levels of markers. Cox regression was used for univariate and multivariable analysis for OS and DFS. To preserve statistical power in subgroup analyses, patients with stage IV disease (n=32) and positive resection margin (n=98) were included in the final analyses. In multivari-

able analyses corrections were made for TNM stage, circumferential margin, age, tumor grade and adjuvant therapy.

RESULTS

HLA class I tumor expression

The analysis of HLA class I expression was performed on 468 stage I-IV rectal cancer patients as, due to staining artifacts and loss of material during the staining procedure, the IHC results of 27 cases could not be analyzed. Representative images of HLA Class I expression are shown in Figure 1. Loss of HLA Class I expression was seen in 70 patients out of 468 patients (15%), down regulation in 105 patients (22 %) and expression was present in the majority of the cases: 293 patients (63%). Patient characteristics and data on HLA class I expression are shown in Table I. Patients with loss of HLA class I tumor expression were diagnosed significantly more often with stage IV tumors (p=0.001) and T3 or T4 tumors (p=0.016). Also, loss of HLA class I was related to more nodal involvement (p=0.003), tumors with poor differentiation (p=0.033) and more adjuvant treatment (p=0.001).

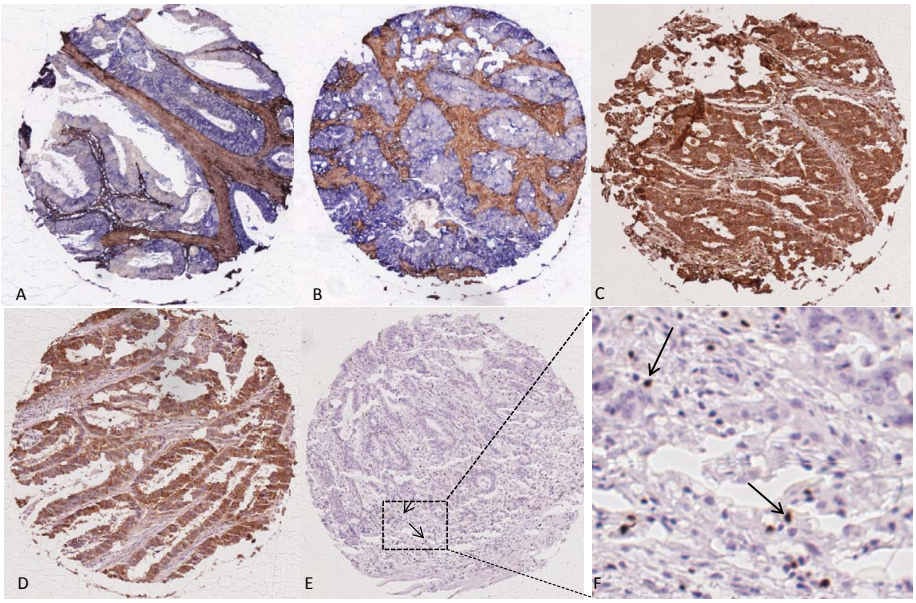


Figure 1: Representative images of HCA2, HC10, HLA-E and –G and Foxp3+ staining in rectal cancer. Representative images of immunohistochemical stainings for HLA Class I expression (HCA2 and HC10), HLA-E and HLA-G expression and presence of Foxp3+ cells, performed according to standard protocols (details in Material and Methods). (A) HCA2-positive tumor (note: positive tumor cells in blue, stromal cells are stained brown);(B) HC10-positive tumor (note: positive tumor cells in blue, stromal cells are stained brown);(C) HLA-E positive tumor (note: positive tumor cells in brown);(D) HLA-G positive tumor (note: positive tumor cells in brown);(E) Presence of Foxp3+ cells (two representative examples of Foxp3+ cells are indicated by arrows) with a magnification in (F).

Table I: Patient Characteristics of the Total Rectal Cancer Cohort and stratified for HLA class I, HLA-G and Foxp3+ expression

	Total population n=495	HLA Class I Loss n=70 (15%)	HLA Class I Downregulation n=105 (22%)	HLA Class I Expression n=293(63%)	HLA-G Weak n=350 (72%)	HLA-G Strong n=134(28%)	Foxp3+ Below median n=240(50%)	Foxp3+ Above median n=238(50%)
Gender (%)								
Male	316 (63.8%)	49 (70.0%)	63 (60.0%)	186 (63.5%)	227 (64.9%)	83 (61.9%)	162 (67.5%)	142 (59.7%)
Female	179 (36.2%)	21 (30%)	42 (40.0%)	107 (36.5%)	123 (35.1%)	51 (38.1%)	78 (32.5%)	96 (40.3%)
Age in years (mean SD)	64.5 (11.3)	64.8 (12.2)	65.5 (11.0)	64.0 (11.0)	64.7 (11.1)	63.9 (11.7)	64.7 (11.9)	(64.2 (10.5)
TNM stage (%)								
I	134 (27.1%)	9 (12.9%)	27 (25.7%)	89 (30.4%)	80 (22.9%)	50 (37.3%)	43 (17.9%)	85 (35.7%)
II	136 (27.5%)	19 (27.1%)	25 (23.8%)	90 (30.7%)	98 (28.0%)	37 (27.6%)	65 (27.1%)	71 (29.8%)
III	193 (39.0%)	33 (47.1%)	51 (48.6%)	96 (32.8%)	146 (41.7%)	41 (30.6%)	112 (46.7%)	72 (30.3%)
IV	32 (6.5%)	9 (12.9%)	2 (1.9%)	18 (6.1%)	26 (7.4%)	6 (4.5%)	20 (8.3%)	10 (4.2%)
Tumor grade (%)								
Moderate	358 (72.3%)	41 (58.6%)	72 (68.9%)	228 (77.8%)	248 (70.9%)	102(76.1%)	160 (66.7%)	185 (77.7%)
Poor	110 (22.2%)	23 (32.9%)	25 (23.8%)	54 (18.4%)	83 (23.7%)	25 (18.7%)	67 (27.9%)	40 (16.8%)
Well	25 (5.1%)	5 (7.1%)	8 (7.6%)	10 (3.4%)	17 (4.9%)	7 (5.2%)	12 (5.0%)	12 (5.0%)
Missing	2 (0.4%)	1 (1.4%)		1 (0.3%)	2 (0.6%)		1 (0.4%)	1 (0.4%)
Adjuvant therapy								
No	402 (81.2%)	46 (65.7%)	84 (80%)	253 (86.3%)	278 (79.4%)	116 (86.6%)	185 (77.1%)	205 (86.1%)
Yes	75 (15.2%)	19 (27.1%)	19 (18.1%)	31 (10.6%)	58 (16.6%)	14 (10.4%)	44 (18.3%)	28 (11.8%)
Missing	18 (3.6%)	5 (7.1%)	2 (1.9%)	9 (3.1%)	14 (4.0%)	4 (3.0%)	11 (4.6%)	5 (2.1%)
Circumferential margin								
Negative	397 (80.2%)	49 (70.0%)	83 (79.0%)	242 (82.6%)	279 (79.7%)	110 (82.1%)	188 (78.3%)	196 (82.4%)
Positive	98 (19.8%)	21 (30.0%)	22 (21.0%)	51 (17.4%)	71 (20.3%)	24 (17.9%)	52 (21.7%)	42 (17.6%)

This table shows the patient characteristics of the entire rectal cancer cohort (n=495) and stratified for HLA class I, HLA-G and Foxp3+ staining.

HLA class I expression was borderline significantly related to a better OS (logrank *p*-value 0.073), but also significantly related to a better DFS (logrank *p*-value 0.012) with a HR of 0.637 (95% CI 0.458-0.886, *p*=0.013) for expression of HLA class I compared to loss of HLA class I expression (Figure 2).

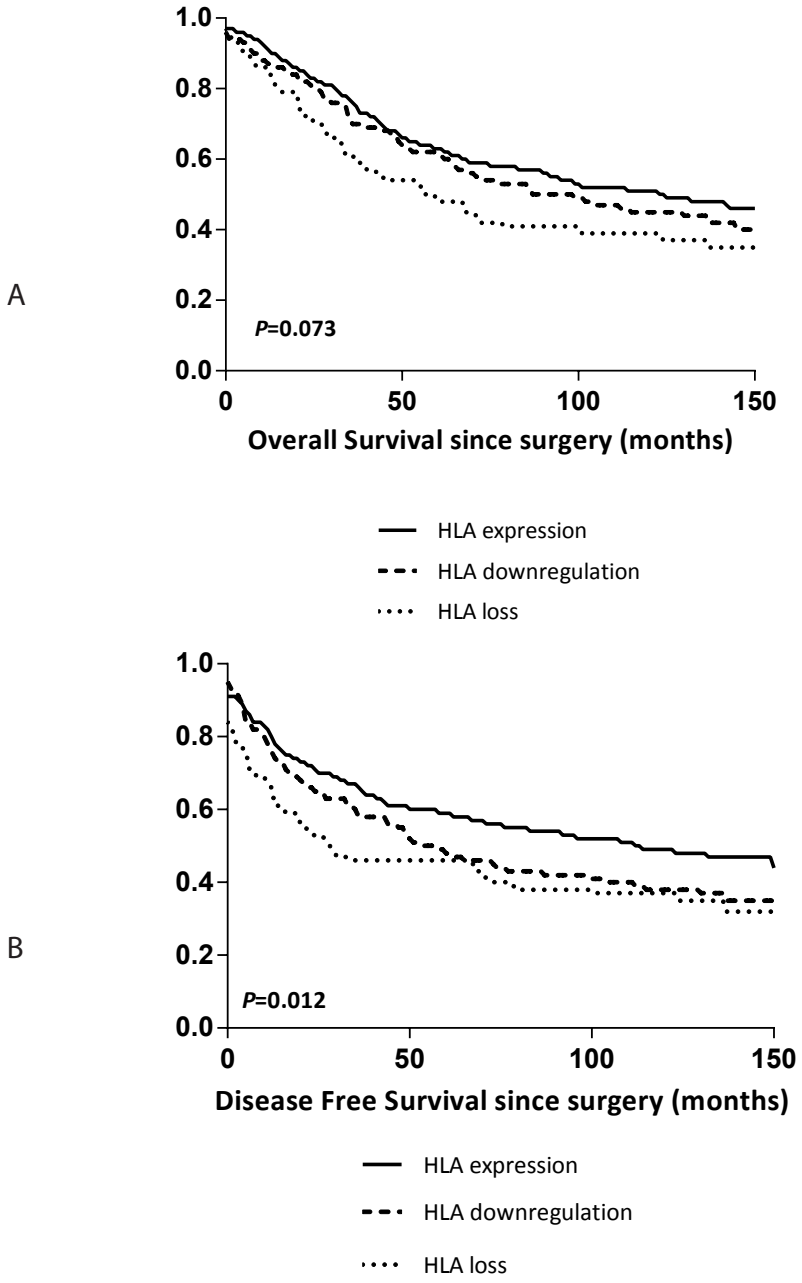


Figure 2: Survival curves stratified for HLA class I tumor expression in rectal cancer.
A) Kaplan Meier curve for Overall Survival in 495 rectal cancer patients stratified for HLA class I tumor expression status. B) Kaplan Meier curve for Disease Free Survival in 495 rectal cancer patients stratified for HLA Class I tumor expression. HLA class I was immunohistochemically determined as described in the Material and Methods section.

Tumor infiltrating Foxp3+ cells

The number of Foxp3+ cells was evaluated in 478 patients, as, due to staining artifacts and loss of material during the staining procedure, the IHC results of 17 cases could not be analyzed. Representative images of Foxp3 staining are shown in Figure 1 and patient characteristics and data on Foxp3+ tumor infiltration are shown in Table I. The mean number Foxp3+ cells per tumor punch was 39 with a median of 27.0. For further analysis Foxp3+ was categorized as below vs. above median due to skewness in the spread of the data. This resulted in 240 patients with presence of Foxp3+ cells below median and 238 patients with presence of Foxp3+ cells above median. Tumors with Foxp3+ cells above median were significantly more often stage I tumors ($p<0.001$), T1 or T2 tumors ($p<0.001$) and showed less nodal involvement ($p<0.001$). Poorly differentiated tumors were associated with tumors with presence of Foxp3+ cells below median ($p=0.022$). Furthermore, tumors with expression of HLA class I showed significantly more Foxp3+ cells above median compared to tumors with loss of HLA class I expression ($p<0.001$).

The presence of Foxp3+ cells above median in the tumor microenvironment was significantly related to a better OS (logrank *p*-value <0.001) and DFS (logrank *p*-value <0.001) with HR's of 0.637 (95% CI 0.500-0.813, $p<0.001$) and 0.624 (95% CI 0.491-0.793, $p<0.001$) respectively in case of presence of Foxp3+ cells above median compared to Foxp3+ cells below median (Figure 3).

HLA-E and HLA-G tumor expression

The analysis of HLA-E and HLA-G was performed on 486 and 484 patients respectively, as, due to staining artifacts and loss of material during the staining procedure, the IHC results of 9 and 11 cases respectively, could not be analyzed. Representative images of non-classical HLA-E and HLA-G immunohistochemical staining results are shown in Figure 1. For HLA-E, 8 patients (1.6%) showed absence of tumor staining, 73 patients (15.0%) showed weak tumor staining, 298 patients (61.3%) showed moderate tumor staining and 107 patients (22.0%) showed strong tumor staining in their punches. For HLA-G, 31 patients (6.4%) had absence of tumor staining, 319 patients (65.9%) had a weak tumor staining, 103 patients (21.3%) had a moderate tumor staining and 31 patients (6.4%) had a strong tumor staining. For analysis the scores were further categorized as weak (absent and weak intensity together) versus strong (moderate and strong intensity together) tumor staining. Strong expression was found in 83.3% (405 out of 486) of the tumors for HLA-E and in 27.7% (134 out of 484) of the tumors for HLA-G expression. Weak expression of HLA-E was significantly related to T4 tumors ($p=0.020$) and more nodal involvement ($p=0.050$). Weak expression of HLA-G was also significantly related to higher tumor stage ($p=0.008$) and more nodal involvement ($p=0.006$). Furthermore, strong expression of HLA-G was significantly associated with presence of Foxp3+ cells

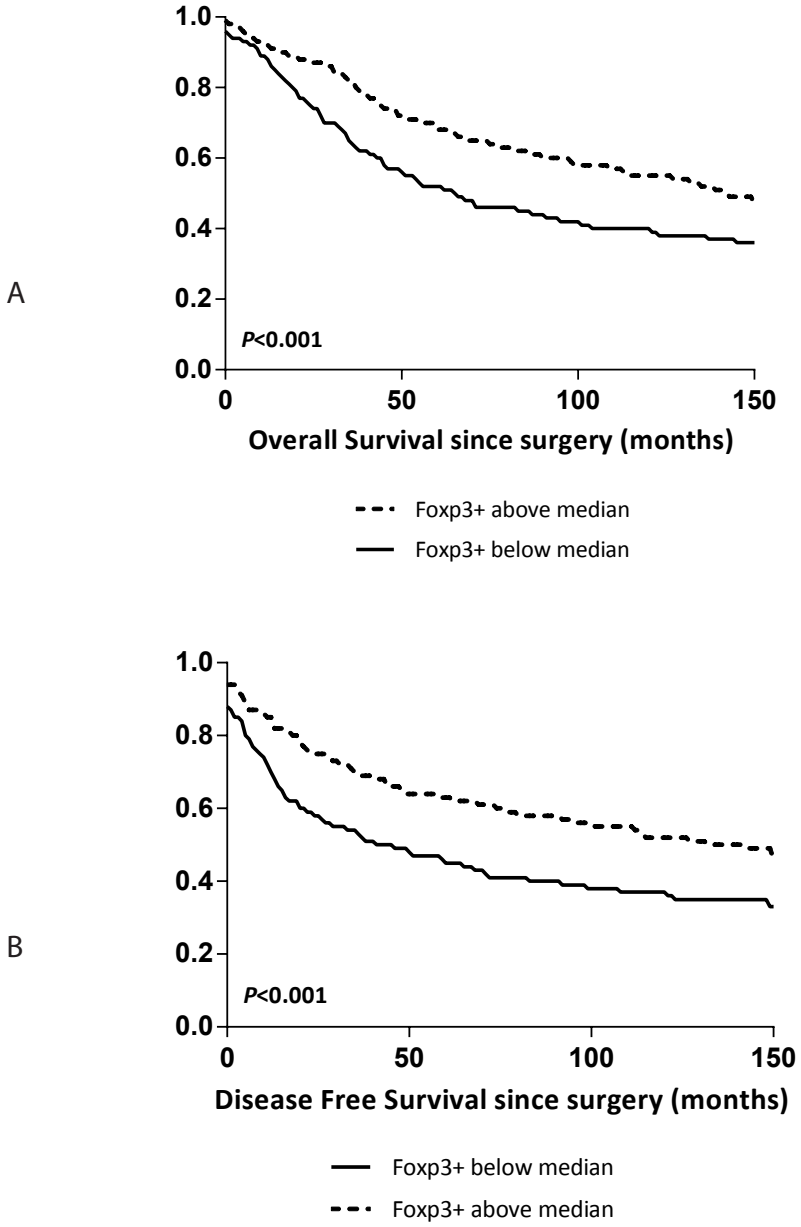


Figure 3: Survival curves stratified for Foxp3+ tumor infiltration in rectal cancer.
A) Kaplan Meier curve for Overall Survival in 495 rectal cancer patients stratified for Foxp3+ tumor infiltration based on the median of the total Foxp3+ infiltration in this cohort. B) Kaplan Meier curve for Disease Free Survival in 495 rectal cancer patients stratified for Foxp3+ tumor infiltration. Foxp3+ tumor infiltration was immunohistochemically determined as described in the Material and Methods section.

above median (p=0.001) and with HLA class I expression (p<0.001). Strong HLA-E was also significantly related to HLA class I expression (p=0.028).

HLA-E expression was not related to OS (p=0.823) or DFS (p=0.784). Strong expression of HLA-G was borderline significantly related to a better OS (logrank p-value 0.056) and significantly related to a better DFS (logrank p-value 0.040) with a HR of 0.753 (95% CI 0.574-0.989, p=0.042) in case of strong expression of HLA-G compared to weak expression of HLA-G.

Multivariable analysis

A multivariable analysis was performed for OS and DFS using the following parameters: age, TNM stage, tumor grade, adjuvant therapy, circumferential margin, HLA class I expression status, HLA-G expression status and Foxp3+ tumor infiltration. Foxp3+ was an independent significant predictor of OS (p=0.018) and DFS (p=0.012). HLA Class I and HLA-G were not significantly related to OS and DFS in multivariable analysis. In Table II all univariate and multivariable analyses are summarized.

Because the type of antibody we used to detect HLA-G expression is known to bind to free heavy chains of classical HLA class I molecules as well [38-40], interaction between these two markers was analysed for survival. In multivariable analysis for OS there was no interaction between HLA-G expression and HLA class I expression (p=0.174). Also, there was no interaction between HLA-G expression and the two types of antibodies used for detection of HLA class I separately; HCA2 expression (p=0.183) and HC10 expression (p=0.461) respectively. For DFS, there was no interaction between HLA-G expression and HLA class I expression as well (p=0.301), neither for HCA2 (p=0.516) nor HC10 (p=0.329).

Analysis of tumor immune-phenotypes

The interaction between tumor cells and immune cells is complex, multifaceted and different interactions are closely linked to each other. In breast- and colon cancer patients, immune subtyping has already shown a promising value in the prediction of prognosis [44;45]. Therefore, we hypothesized that combined analysis of immune markers may better reflect patients' outcome as a result of interaction between tumor cells and the immune system in rectal cancer as well. We have shown above that patients with tumors showing expression of HLA class I, expression of HLA-G and presence of Foxp3+ cell infiltration above median showed better survival outcomes when analyzed separately. HLA-E tumor expression was not related to survival. Based on the prognostic value of the individual markers, a score was created for the combination of HLA class I, HLA-G and Foxp3+. HLA class I was divided into 3 scores, which ranged from 0 for loss of expression to 2 for high expression. HLA-G and Foxp3+ were divided into 2 scores; 0 for weak HLA-G expression or Foxp3+ below median and 1 for strong HLA-G expression

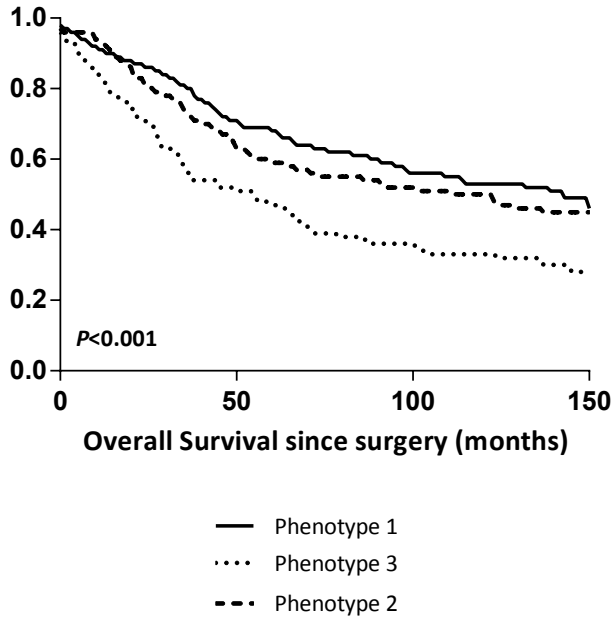
or Foxp3+ above median. Combining the scores of the individual markers resulted in a scoring range from 0 to 4. The entire population was divided into 3 tumor immune-phenotypes: patients with scores 3 and 4 (phenotype 1, n=210), patients with score 2 (phenotype 2, n=139) and patients with scores 0 and 1 (phenotype 3, n=112).

In survival analyses, these phenotypes showed significant differences in patient outcome. Survival outcome increased with an increasing number of positive prognostic immune markers expressed in the tumor. Patients with phenotype 3 showed a significantly worse OS (logrank $p<0.001$) and DFS (logrank $p<0.001$) with HR's of 1.88 (95% CI 1.40-2.53, p<0.001) for OS and 2.06 (95% CI 1.54-2.75, $p<0.001$) for DFS, when compared to phenotype 1 (Figure 4).

Multivariable analysis of the tumor immune-phenotypes

For the tumor immune-phenotype, univariate analysis and multivariable analysis was also performed to determine OS and DFS as written above. In univariate analysis the immune-phenotype was a significant predictor of OS ($p<0.001$) and DFS ($p<0.001$) (Table II). In multivariable analysis the immune-phenotype was an independent predictor of DFS ($p=0.019$). It was not an independent predictor of OS ($p=0.122$). When compared to the multivariable analyses of the individual immune markers as shown in table II, the combination between immune markers, the tumor immune-phenotype, showed a stronger and additive prognostic potential, indicating a complex and multifaceted interaction between tumor cells and immune cells.

A



B

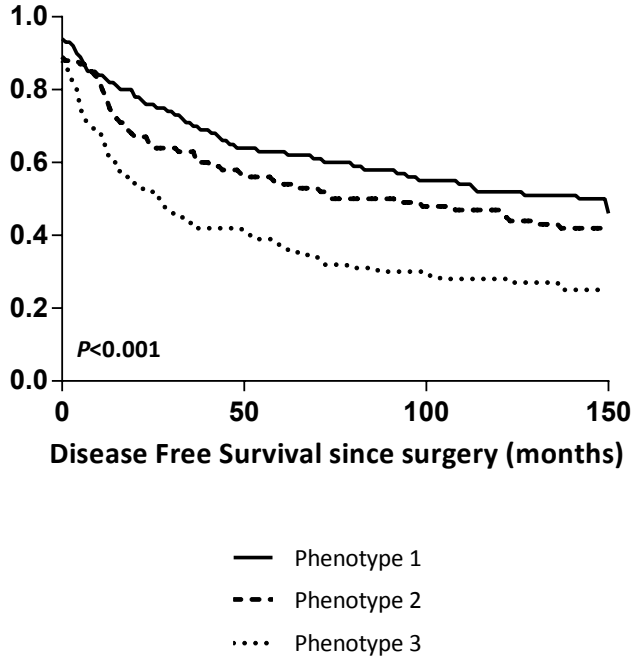


Figure 4: Survival curves stratified for immune-phenotypes in rectal cancer.
A) Kaplan Meier curve for Overall Survival in 495 rectal cancer patients stratified for all the different combinations between tumor expression of HLA class I, HLA-G and the presence of Foxp3+ cells based on which 3 immune-phenotypes could be distinguished. See results section for explanation of the phenotypes. B) Kaplan Meier curve for Disease Free Survival in 495 rectal cancer patients stratified for all the different combinations between tumor expression of HLA class I, HLA-G and the presence of Foxp3+ cells based on which 3 immune phenotypes could be distinguished. See results section for explanation of the phenotypes.

Table II: Univariate and multivariable analyses of Disease Free Survival (DFS) and Overall Survival (OS) for the different immune markers and for tumor immune phenotypes

	DFS						OS					
	Univariate analysis			Multivariable analysis**			Univariate analysis			Multivariable analysis**		
	HR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value
HLA class I			0.013*			0.548			0.075			0.984
Loss	1.00			1.00			1.00			1.00		
Downregulation	0.84	0.58-1.22		1.08	0.72-1.62		0.78	0.53-1.15		1.04	0.68-1.59	
Expression	0.64	0.46-0.89		0.91	0.63-1.32		0.68	0.49-0.95		1.03	0.70-1.52	
Foxp3+			<0.001*			0.012*			<0.001*			0.018*
Below median	1.00			1.00			1.00			1.00		
Above median	0.62	0.49-0.79		0.72	0.56-0.93		0.64	0.50-0.81		0.73	0.56-0.95	
HLA-G			0.042*			0.849			0.056			0.418
Weak expression	1.00			1.00			1.00			1.00		
Strong expression	0.75	0.57-0.99		0.85	0.63-1.13		0.76	0.58-1.01		0.88	0.66-1.19	
Immune phenotype			<0.001*			0.019*			<0.001*			0.122
Phenotype 1	1.00			1.00			1.00			1.00		
Phenotype 2	1.26	0.94-1.68		1.13	0.83-1.54		1.18	0.87-1.58		1.07	0.78-1.47	
Phenotype 3	2.06	1.54-2.75		1.56	1.14-2.14		1.88	1.40-2.53		1.39	1.01-1.92	
TNM stage			<0.001*						<0.001*			
I-II	1.00						1.00					
III-IV	2.65	2.09-3.37					2.65	2.08-3.38				
Circumferential margin			<0.001*						<0.001*			
Negative	1.00						1.00					
Positive	2.26	1.73-2.94					2.42	1.85-3.16				
Age (continuous)	1.03	1.02-1.04	<0.001*				1.04	1.03-1.05	<0.001*			
Tumor grade			0.094						0.058			
Well	1.00						1.00					
Moderate	0.88	0.53-1.46					0.87	0.52-1.44				
Poor	1.19	0.69-2.04					1.21	0.70-2.09				
Adjuvant chemotherapy			<0.001*						<0.001*			
No	1.00						1.00					
Yes	2.38	1.77-3.20					2.43	1.80-3.28				

** Statistical significance*

*** Corrected for TNM stage, circumferential margin, age, tumor grade and adjuvant therapy*

DISCUSSION

In this study, by combining the immune-related tumor markers HLA class I, HLA-G and Foxp3+, we reported an independent association between tumor immune-phenotype and patient outcome. These phenotypes might represent how the immune system controls tumor growth and metastases in rectal cancer.

Previous studies on HLA class I expression, which focused on a mixed population of colon- and rectal cancer together, have shown inconsistent findings [13;14]. Our study showed a survival benefit for patients with tumors expressing HLA class I. These results are partly comparable with results from Watson *et al*., who showed that low expression of HLA class I was related to a poor prognosis in a large group of colorectal cancer patients, whereas tumors with loss or expression of HLA class I were associated with a survival benefit [14]. A substantial part of Watson's cohort showed HLA class I negative tumors (24.6%). In our cohort, consisting solely of rectal cancer patients, only 15.0% of the patients had tumors with loss of HLA class I expression, which might indicate that colon cancers lose their HLA class I expression more often. Previously, Speetjens *et al.* investigated the prognostic value of HLA class I expression in rectal cancer patients from the Dutch TME Trial as well [27]. In this study, as described in the methods sections, a new TMA was used without complete overlap and thus different patients. Both studies showed a survival benefit for patients with tumors showing expression of HLA class I. Because we have changed the scoring criteria based on recommendation by the International HLA and Immunogenetics Workshop [42] differences have to be acknowledged. Speetjens *et al.* reported that 16% of non-irradiated patients had tumors with loss and downregulation of HLA Class I, whereas our study showed 37% (15% loss and 22% downregulation). Thus, besides a different patient cohort, other possible explanations for inconsistent findings between studies are the use of different definitions of HLA class I expression and differences in staining techniques. Furthermore, tumor microsatellite status might also play an important role. Approximately 50% of all proximal colon tumors show microsatellite instability (MSI), whereas almost all distal colon and rectal cancers are microsatellite stable (MSS) tumors [46;47]. Loss of HLA class I has been described more significantly in MSI colorectal tumors compared to MSS right-sided colon tumors [48;49]. HLA class I negative tumors are therefore more likely to be MSI tumors with a different clinical behavior than MSS colorectal tumors [27]. Since MSI tumors have a better prognosis, MSI might influence prognostic results when considering HLA class I expression in colorectal tumors [46]. In this rectal cancer cohort determination of the microsatellite status would not have been useful. Research has shown that in only 2% of rectal cancers MSI can be found [50], resulting in insufficient statistical power for separate analyses Finally, colon and rectum are biologically different tissues; the colon epithelium consists of simple columnar epithelium, whereas the rectum is a transition from single columnar epithelium to stratified

squamous epithelium, which might result in different outcomes. The Cancer Genome Atlas Network attempted to find biological differences between colon and rectal cancer. However, only differences in anatomical tumor site with more hypermethylation in right-sided tumors were found, which might be explained by different embryonic origins of the right-and left-sided tumors [28].

Results in our study regarding non-classical HLA-G are remarkable. HLA-G expression can inhibit NK-cells from lysing tumor cells that have lost or downregulated classical HLA class I expression as a secondary immune escape [51;52]. However, in this study, positive HLA-G expression was correlated with a longer disease free survival.

The antibody used to stain HLA-G can also bind to free heavy chains of classical HLA class I molecules as well, possibly explaining the remarkable results. We therefore performed an interaction analysis between these antibodies. However, no interaction between HLA-G and HLA class I expression was found. Furthermore, HLA-G is found to be highly immunosuppressive by directly inhibiting NK cells, but also by recruitment of Tregs and induction of Treg differentiation [53]. Our study showed that strong HLA-G expression was significantly related to presence of more Foxp3+ cells, possibly explaining the favourable prognosis of tumors with strong HLA-G expression, since tumors that attracted more Foxp3+ cells had a better outcome in our cohort. Immune regulation in cancer still remains complex and multifaceted, and not all immune related mechanisms are completely clear. To our knowledge, no other studies on HLA-E and HLA-G are performed on rectal cancer specifically and therefore no other comparisons could be made.

The presence of Foxp3+ cells in the tumor microenvironment is thought to inhibit host-protective antitumor responses and especially CTL activity [6]. A high density of tumor infiltrating Foxp3+ cells has shown to be associated with an unfavorable prognosis in a wide range of human carcinomas [54;55]. However, in accordance with our results, opposite results are described in CRC [20;21]. A possible explanation could be a significant association between HLA class I tumor expression and Foxp3+ tumor infiltration in our cohort. Foxp3+ infiltrating cells might be necessary to counteract CTL activity in tumors expressing HLA class I to prevent an auto-immune response on other bodily cells as well. Another explanation might be a different micro-environment of rectal cancer, which is colonized with many gastro-intestinal bacteria, triggering the production of pro-inflammatory cytokines causing tumor-enhancing effects. Instead of the specificity of infiltrating T-cells for tumor-antigens, T-cells in rectal cancer could be more specific for the microflora and suppress inflammation and immune responses from bacterial invasion, resulting in an anti-tumorigenic effect [56].

As shown in our results and results from our previous studies in breast cancer, immune markers are related to each other [31;32]. Studying solely one marker might not be enough to truly understand cancer immune surveillance. When we combined our markers, patients showing the worst prognosis were patients with tumors bearing 2 or 3 nega-

tive prognostic markers; patients with loss of HLA class I tumor expression, weak HLA-G tumor expression and low tumor infiltration with Foxp3+ cells. These patients therefore qualify as very low immune susceptible. They probably were able to elicit only a minimal CTL attack and subsequently attracted little to no Foxp3+ cells in their tumor micro-environment, possibly explaining their worse prognosis. Furthermore, patients with tumors showing loss of HLA class I expression, low Foxp3+ cell infiltration and strong HLA-G expression showed the worst outcome perspectives. These patients probably had tumors which were highly 'edited' as well, causing a minimal CTL attack and subsequently attracted little to no Foxp3+ cells, and because of strong HLA-G expression were able to escape further immune recognition through inhibition of NK cell recognition and subsequently no elimination [51;52].

CONCLUSIONS

In conclusion, we were able to identify local immune escape mechanisms of rectal cancer, where the presence of Foxp3+ infiltration greatly influences a better prognosis. Loss of HLA class I expression, weak non-classical HLA-G expression and the presence of Foxp3+ below median were related to a worse outcome. Combining these immune-related markers identified 3 groups, which were highly selective and discriminative regarding patient outcome. Prognosis increased with a decrease in negative prognostic markers. In the future these findings might contribute to better treatment allocation.

REFERENCE LIST

(1) Halvorsen TB, Seim E. Association between invasiveness, inflammatory reaction, desmoplasia and survival in colorectal cancer. *J Clin Pathol* 1989;42:162-166.

(2) Menon AG, Janssen-van Rhijn CM, Morreau H et al. Immune system and prognosis in colorectal cancer: a detailed immunohistochemical analysis. *Lab Invest* 2004;84:493-501.

(3) Murphy J, O'Sullivan GC, Lee G et al. The inflammatory response within Dukes' B colorectal cancers: implications for progression of micrometastases and patient survival. *Am J Gastroenterol* 2000;95:3607-3614.

(4) Naito Y, Saito K, Shiiba K et al. CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. *Cancer Res* 1998;58:3491-3494.

(5) Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646-674.

(6) Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 2011;331:1565-1570.

(7) Cavallo F, De GC, Nanni P, Forni G, Lollini PL. 2011: the immune hallmarks of cancer. *Cancer Immunol Immunother* 2011;60:319-326.

(8) Garrido F, Cabrera T, Concha A, Glew S, Ruiz-Cabello F, Stern PL. Natural history of HLA expression during tumour development. *Immunol Today* 1993;14:491-499.

(9) Marincola FM, Jaffee EM, Hicklin DJ, Ferrone S. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Adv Immunol* 2000;74:181-273.

(10) Hokland M, Kuppen PJ. Natural killer cells: from "disturbing" background to central players of immune responses. *Mol Immunol* 2005;42:381-383.

(11) Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nat Immunol* 2002;3:999-1005.

(12) Marin R, Ruiz-Cabello F, Pedrinaci S et al. Analysis of HLA-E expression in human tumors. *Immunogenetics* 2003;54:767-775.

(13) Menon AG, Morreau H, Tollenaar RA et al. Down-regulation of HLA-A expression correlates with a better prognosis in colorectal cancer patients. *Lab Invest* 2002;82:1725-1733.

(14) Watson NF, Ramage JM, Madjd Z et al. Immunosurveillance is active in colorectal cancer as down-regulation but not complete loss of MHC class I expression correlates with a poor prognosis. *Int J Cancer* 2006;118:6-10.

(15) Palmisano GL, Contardi E, Morabito A, Gargaglione V, Ferrara GB, Pistillo MP. HLA-E surface expression is independent of the availability of HLA class I signal sequence-derived peptides in human tumor cell lines. *Hum Immunol* 2005;66:1-12.

(16) Wischhusen J, Waschbisch A, Wiendl H. Immune-refractory cancers and their little helpers—an extended role for immunetolerogenic MHC molecules HLA-G and HLA-E? *Semin Cancer Biol* 2007; 17:459-468.

(17) Nosho K, Baba Y, Tanaka N et al. Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer, and prognosis: cohort study and literature review. *J Pathol* 2010;222:350-366.

(18) Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat Rev Immunol* 2006;6:715-727.

(19) Deng L, Zhang H, Luan Y et al. Accumulation of foxp3+ T regulatory cells in draining lymph nodes correlates with disease progression and immune suppression in colorectal cancer patients. *Clin Cancer Res* 2010;16:4105-4112.

(20) Salama P, Phillips M, Grieu F et al. Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol* 2009;27:186-192.

(21) Suzuki H, Chikazawa N, Tasaka T et al. Intratumoral CD8(+) T/FOXP3 (+) cell ratio is a predictive marker for survival in patients with colorectal cancer. *Cancer Immunol Immunother* 2010;59:653-661.

(22) Zeestraten EC, Van Hoesel AQ, Speetjens FM et al. FoxP3- and CD8-positive Infiltrating Immune Cells Together Determine Clinical Outcome in Colorectal Cancer. *Cancer Microenviron* 2011.

(23) Sandel MH, Speetjens FM, Menon AG et al. Natural killer cells infiltrating colorectal cancer and MHC class I expression. *Mol Immunol* 2005;42:541-546.

(24) Bossard C, Bezieau S, Matysiak-Budnik T et al. HLA-E/beta2 microglobulin overexpression in colorectal cancer is associated with recruitment of inhibitory immune cells and tumor progression. *Int J Cancer* 2012;131:855-863.

(25) Ye SR, Yang H, Li K, Dong DD, Lin XM, Yie SM. Human leukocyte antigen G expression: as a significant prognostic indicator for patients with colorectal cancer. *Mod Pathol* 2007;20:375-383.

(26) de Bruin EC, van de Velde CJ, van Krieken JH, Marijnen CA, Medema JP. Epithelial human leukocyte antigen-DR expression predicts reduced recurrence rates and prolonged survival in rectal cancer patients. *Clin Cancer Res* 2008;14:1073-1079.

(27) Speetjens FM, de Bruin EC, Morreau H et al. Clinical impact of HLA class I expression in rectal cancer. *Cancer Immunol Immunother* 2008;57:601-609.

(28) Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 2012;487:330-337.

(29) Kapiteijn E, Liefers GJ, Los LC et al. Mechanisms of oncogenesis in colon versus rectal cancer. *J Pathol* 2001;195:171-178.

(30) Li JN, Zhao L, Wu J et al. Differences in gene expression profiles and carcinogenesis pathways between colon and rectal cancer. *J Dig Dis* 2012;13:24-32.

(31) de Kruijf EM, van Nes JG, Sajet A et al. The predictive value of HLA class I tumor cell expression and presence of intratumoral Tregs for chemotherapy in patients with early breast cancer. *Clin Cancer Res* 2010;16:1272-1280.

(32) de Kruijf EM, Sajet A, van Nes JG et al. HLA-E and HLA-G expression in classical HLA class I-negative tumors is of prognostic value for clinical outcome of early breast cancer patients. *J Immunol* 2010;185:7452-7459.

(33) Kapiteijn E, Kranenbarg EK, Steup WH et al. Total mesorectal excision (TME) with or without preoperative radiotherapy in the treatment of primary rectal cancer. Prospective randomised trial with standard operative and histopathological techniques. Dutch ColoRectal Cancer Group. *Eur J Surg* 1999;165:410-420.

(34) Nagtegaal ID, van de Velde CJ, van der Worp E, Kapiteijn E, Quirke P, van Krieken JH. Macroscopic evaluation of rectal cancer resection specimen: clinical significance of the pathologist in quality control. *J Clin Oncol* 2002;20:1729-1734.

(35) Generali D, Bates G, Berruti A et al. Immunomodulation of FOXP3+ regulatory T cells by the aromatase inhibitor letrozole in breast cancer patients. *Clin Cancer Res* 2009;15:1046-1051.

(36) Hill JA, Feuerer M, Tash K et al. Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity* 2007;27:786-800.

(37) Lo ME, Sibilio L, Melucci E et al. HLA-E: strong association with beta2-microglobulin and surface expression in the absence of HLA class I signal sequence-derived peptides. *J Immunol* 2008;181:5442-5450.

(38) Menier C, Saez B, Horejsi V et al. Characterization of monoclonal antibodies recognizing HLA-G or HLA-E: new tools to analyze the expression of nonclassical HLA class I molecules. *Hum Immunol* 2003;64:315-326.

(39) Polakova K, Kuba D, Russ G. The 4H84 monoclonal antibody detecting beta2m free nonclassical HLA-G molecules also binds to free heavy chains of classical HLA class I antigens present on activated lymphocytes. *Hum Immunol* 2004;65:157-162.

(40) Zhao L, Teklemariam T, Hantash BM. Reassessment of HLA-G isoform specificity of MEM-G/9 and 4H84 monoclonal antibodies. *Tissue Antigens* 2012;80:231-238.

(41) Viera AJ, Garrett JM. Understanding interobserver agreement: the kappa statistic. *Fam Med* 2005; 37:360-363.

(42) Chew SF, Kanaan C, Tait BD. HLA expression and cancer—14th IHIWS immunohistochemistry quality control exercise exchange results. *Tissue Antigens* 2007;69 Suppl 1:248-251.

(43) Benevolo M, Mottolese M, Tremante E et al. High expression of HLA-E in colorectal carcinoma is associated with a favorable prognosis. *J Transl Med* 2011;9:184.

(44) Zeestraten EC, Reimers MS, Saadatmand S et al. Combined analysis of HLA class I, HLA-E and HLA-G predicts prognosis in colon cancer patients. *Br J Cancer* 2013.

(45) de Kruijf EM, Engels CC, van de Water W et al. Tumor immune subtypes distinguish tumor subclasses with clinical implications in breast cancer patients. *Breast Cancer Res Treat* 2013;142: 355-364.

(46) Popat S, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. *J Clin Oncol* 2005;23:609-618.

(47) Soreide K, Janssen EA, Soiland H, Korner H, Baak JP. Microsatellite instability in colorectal cancer. *Br J Surg* 2006;93:395-406.

(48) Dierssen JW, de Miranda NF, Ferrone S et al. HNPCC versus sporadic microsatellite-unstable colon cancers follow different routes toward loss of HLA class I expression. *BMC Cancer* 2007;7:33.

(49) Kloor M, Becker C, Benner A et al. Immunoselective pressure and human leukocyte antigen class I antigen machinery defects in microsatellite unstable colorectal cancers. *Cancer Res* 2005;65: 6418-6424.

(50) Slattery ML, Curtin K, Wolff RK et al. A comparison of colon and rectal somatic DNA alterations. *Dis Colon Rectum* 2009;52:1304-1311.

(51) Bukur J, Jasinski S, Seliger B. The role of classical and non-classical HLA class I antigens in human tumors. *Semin Cancer Biol* 2012;22:350-358.

(52) Lin A, Zhang X, Xu HH, Xu DP, Ruan YY, Yan WH. HLA-G expression is associated with metastasis and poor survival in the Balb/c nu/nu murine tumor model with ovarian cancer. *Int J Cancer* 2012; 131:150-157.

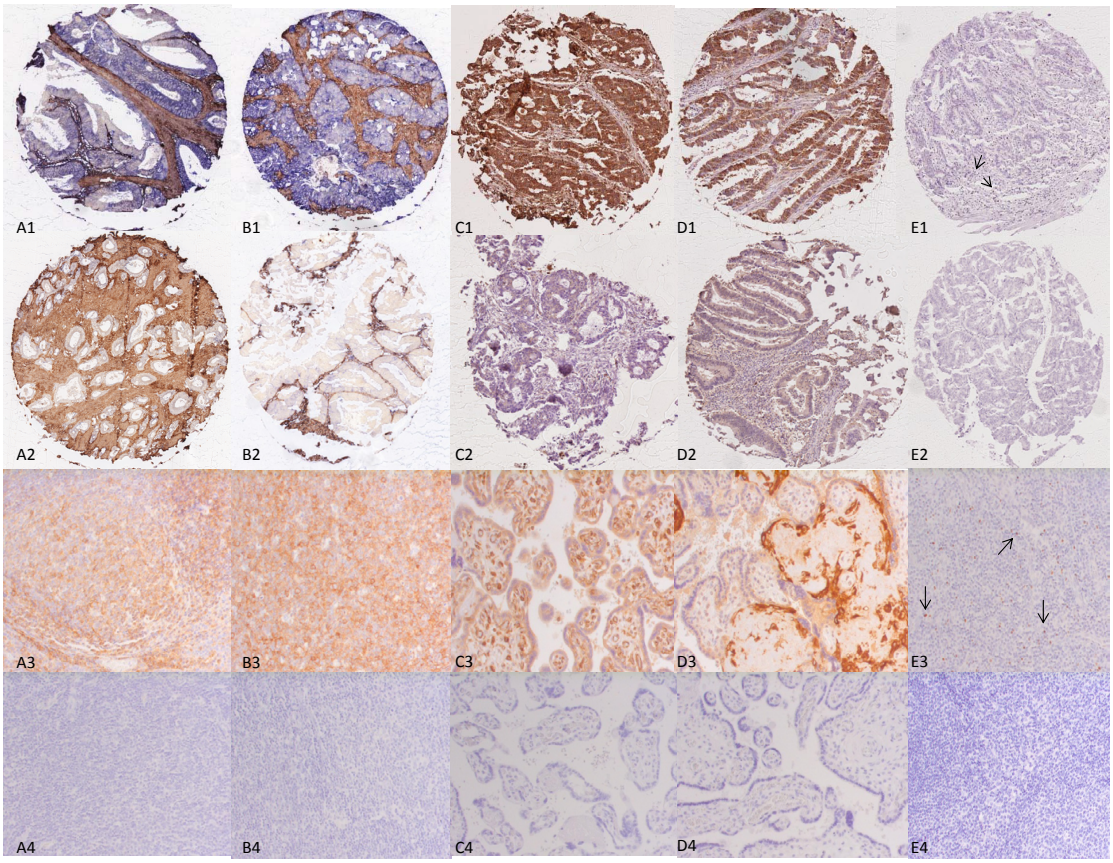
(53) Du L, Xiao X, Wang C et al. Human leukocyte antigen-G is closely associated with tumor immune escape in gastric cancer by increasing local regulatory T cells. *Cancer Sci* 2011;102:1272-1280.

(54) Bates GJ, Fox SB, Han C et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol* 2006;24:5373-5380.

(55) Petersen RP, Campa MJ, Sperlazza J et al. Tumor infiltrating Foxp3+ regulatory T-cells are associated with recurrence in pathologic stage I NSCLC patients. *Cancer* 2006;107:2866-2872.

(56) Ladoire S, Martin F, Ghiringhelli F. Prognostic role of FOXP3+ regulatory T cells infiltrating human carcinomas: the paradox of colorectal cancer. *Cancer Immunol Immunother* 2011;60:909-918.

SUPPLEMENTAL MATERIAL



Supplemental Figure 1: Representative images of HCA2, HC10, HLA-E, HLA-G and Foxp3+ staining in rectal cancer.

Representative images of immunohistochemical stainings with positive and negative controls for HLA Class I expression (HCA2 and HC10), HLA-E and HLA-G expression and presence of Foxp3+ cells, performed according to standard protocols (details in Material and Methods section). (A) HCA2 expression, positive tumor (note: positive tumor cells in blue, stromal cells are stained brown) (A1), negative tumor (A2), tonsil which served as positive control (A3), tonsil which underwent the whole immuno-histochemical staining without primary antibody served as negative control (A4); (B) HC10 expression, positive tumor (note: positive tumor cells in blue, stromal cells are stained brown) (B1), negative tumor (B2), tonsil which served as positive control (B3), tonsil which underwent the whole immuno-histochemical staining without primary antibody served as negative control (B4); (C) HLA-E expression, positive tumor (note: positive tumor cells are stained brown) (C1), negative tumor (C2), placenta which served as positive control (C3), placenta which underwent the whole immuno-histochemical staining without primary antibody served as negative control (C4); (D) HLA-G expression, positive tumor (note: positive tumor cells are stained brown) (D1), negative tumor (D2), placenta which served as positive control (D3), placenta which underwent the whole immuno-histochemical staining without primary antibody served as negative control (D4); (E) Presence of Foxp3+ cells, tumor with presence of Foxp3+ cells (indicated by arrows) (E1), tumor with absence of Foxp3+ cells (E2), tonsil which served as positive control for Foxp3+ cells (indicated by arrows) (E3), tonsil which underwent the whole immuno-histochemical staining without primary antibody served as negative control (E4).

CHAPTER 4

Combined analysis of biomarkers of proliferation and apoptosis in colon cancer; an immunohistochemistry based study using tissue microarray

Marlies S. Reimers*, Eliane C.M. Zeestraten*, Tert C. van Alphen, Jan-Willem T. Dekker, Hein Putter, Sepideh Saadatmand, Gerrit Jan Liefers, Cornelis J.H. van de Velde, Peter J.K. Kuppen

*Both authors contributed equally to this work

International Journal of Colorectal Disease, 2014 Sep; 29(9):1043-52

ABSTRACT

Background

Disturbance of the balance between proliferation and apoptosis is an important hallmark of tumor development. The goal of this study was to develop a descriptive parameter that represents this imbalance and relate this parameter to clinical outcome in all four stages of colon cancer.

Methods

The study population consisted of 285 stage I-IV colon cancer patients of which a tumor tissue micro array (TMA) was available. TMA sections were immunohistochemically stained and quantified for presence of Ki67 and cleaved caspase-3 tumor expression. These results were used to develop the combined apoptosis proliferation (CAP) parameter and correlated to patient outcome.

Results

The CAP parameter was significantly related to clinical outcome; patients with CAP ++ (high level of both apoptosis and proliferation) showed the best outcome perspectives (Overall Survival (OS), p=0.004 and Disease Free Survival (DFS), p=0.009). The effect of the CAP parameter was related to tumor microsatellite status, and indirectly to tumor location, where left-sided tumors with CAP + - (high level of proliferation, low level of apoptosis) showed a worse prognosis (DFS p-value 0.02) and right-sided tumors with CAP + - had a better prognosis (DFS p-value 0.032). With stratified analyses, the CAP parameter remained significant in stage II tumors only.

Conclusions

The CAP parameter, representing outcome of the balance between the level of apoptosis and proliferation, can be used as a prognostic marker in colon cancer patients for both DFS and OS, particularly in left sided, microsatellite stable tumors when TNM stage is taken into account.

INTRODUCTION

A key factor in tissue homeostasis, especially of the intestinal mucosa, is the balance that exists between the level of cell death and the level of cell proliferation [1-3]. Two important hallmarks of the process of tumorigenesis are responsible for disturbance of this balance and therefore contribute to the initiation and maintenance of tumor growth and development [4;5]. These hallmarks are: deregulation of the proliferative signaling pathway and deregulation of the pathway of apoptosis [4]. Both result in either non- or malfunction of important enzymes or unrestricted release of growth-promoting signals that under normal circumstances are necessary to maintain tissue homeostasis [5-12]. The level of cell proliferation and apoptosis can be studied with immunohistochemistry (IHC) taking advantage of all of the benefits of this technique, such as speed, routine availability, low costs, and high level of automation. The level of apoptosis can be evaluated through staining specifically the activated, cleaved form of the pro-apoptotic enzyme caspase-3 in the tumor cell cytoplasm. Caspase-3 is the final enzyme to become activated in the caspase cascade, which is the common pathway in the execution of apoptosis after the intrinsic and extrinsic apoptosis induction pathways converge. Therefore, the expression level of activated or cleaved caspase-3 should give a reliable measure of the level of apoptosis [13]. The proliferation activity of a tumor can be estimated by determining the expression levels of specific cell cycle-related proteins also by using IHC. A widely used marker is the Ki67 antigen, which is expressed in nuclei during all cell cycle phases except during the G_0 phase [14]. Previous studies showed contradicting results with respect to the relation of the level of apoptosis or proliferation in tumor resection specimens and patient outcome in colon cancer [15-22]. We hypothesize that, because tissue homeostasis depends on the balance between cell death and proliferation levels, the level of disbalance between these processes indicate tumor aggressiveness. Therefore, combined and not separate analysis of these parameters might be of prognostic relevance in colon cancer patients.

In this study we determined both the level of tumor cell apoptosis and proliferation in resection specimens of a large cohort of colon cancer patients. We combined the results into one parameter and related this parameter to patient outcome data.

MATERIALS AND METHODS

Patients and tumors

The patient cohort consisted of 470 colorectal cancer patients treated with surgery for their primary tumor in the LUMC between 1991 and 2001. Clinico-pathological and follow-up data were collected retrospectively from hospital records and the hospital's

oncology database. This research was performed according to the code of conduct for responsible use.

Patient records information was anonymized and de-identified prior to analysis according to national ethical guidelines ("Code for Proper Secondary Use of Human Tissue", Dutch Federation of Medical Scientific Societies). Patients with a history of cancer other than basal cell carcinoma or cervical carcinoma *in situ*, patients that received radio- and/or chemotherapy treatment prior to resection, patients with multiple synchronous colon tumors, and patients with rectal cancers were excluded from the analysis (n=185). The entire study cohort consisted of 285 patients. Right-sided tumors were defined as those originating proximal to the splenic flexure and left-sided as those originating distal to the splenic flexure.

Primary Antibodies

The following antibodies were used in the immunohistochemical stainings: Mouse monoclonal antibody anti-Ki67 (DAKO Glostrup Denmark Art.M7240 clone MIB-1) to determine the level of tumor cell proliferation and rabbit polyclonal antibody anti-ASP-175 (Cell signaling Danvers, USA, Art.9661) was used for cleaved caspase-3 identification to determine the level of apoptosis.

Immunohistochemistry

Qualified pathologist evaluated the tumor material from all patients included for histopathological characteristics according to current standards during the routine hospital diagnostic process. Formalin-fixed paraffin-embedded tumor blocks of the primary tumor were collected from the pathology department. Sections were cut for haematoxylin and eosin staining, and representative tumor regions based on histological assessment were used for preparation of tumor tissue microarray (TMA) blocks. From each donor block, three 0.6 mm tissue cores were punched from tumor areas and transferred into a recipient paraffin block using a custom-made precision instrument.

Immunohistochemistry (IHC) staining was performed on 4 µm sections that were cut from each receiver block and mounted on glass. For each primary antibody, all slides were stained simultaneously to avoid inter-assay variation. Tissue sections were deparaffinized and rehydrated. For antigen retrieval, 0.01 M EDTA buffer (pH 8) was used for 10 minutes at maximum power in a microwave oven for anti-Ki67. Citrate buffer 0.1M (pH 6) was used for anti-ASP-175. Endogenous peroxidase was blocked for 20 minutes in 0.3% hydrogen peroxide in methanol. Sections were incubated overnight with either anti-Ki67 or anti-ASP-175 at predetermined optimal dilutions. After 30 minutes of incubation with Envision anti-mouse (K4001; DAKO Cytomation, Glostrup, Denmark) or Envision anti-rabbit (K4003); DAKO Cytomation, Glostrup, Denmark), sections were visualized using diaminobenzidine solution. Tissue sections were counterstained with haematoxylin,

dehydrated and finally mounted in malinol. Sections with phosphate buffer saline (PBS) instead of primary antibody, which underwent the complete staining protocol served as negative controls.

Evaluation of immunohistochemistry

Microscopic analyses of Ki67 and cleaved caspase-3 expression was performed by two independent observers (M.S.R: 100% and T.C.A.: 30%) in a blinded manner. For Ki67, the percentage of tumor cells that showed nuclear staining was assessed. For determination of tumor cell apoptosis, the absolute number of caspase-3 expressing tumor cells in each tumor punch that showed cytoplasmatic and perinuclear staining was counted. The Cohen's Kappa for inter observer variability was 0.73 and 0.6 for Ki67 and cleaved caspase-3 respectively. Therefore, there was substantial agreement between the two observers and all scores were averaged. For analysis a cut-off at the median was chosen, dividing the samples in low (<27% positive tumor cells) or high nuclear Ki67 expression (≥27%). The use of this percentage of positive cells as a cut-off point is supported by Fluge *et al.* [18]. Cleaved caspase-3 was quantified into two categories of IHC cytoplasmatic tumor staining levels. Negative staining; implied no positive tumor cells in either of the three cores, in all other cases the staining was denoted as positive. Representative images of the Ki67 and caspase-3 staining are shown in Figure 1.

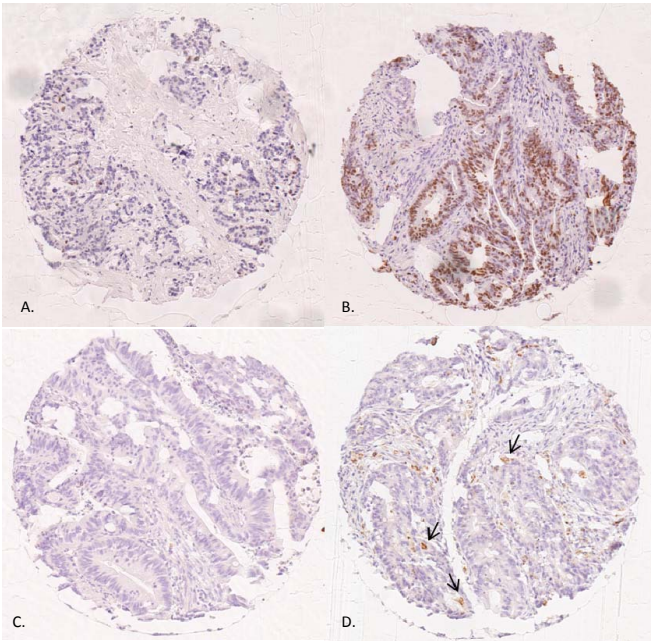


Figure 1: Representative images of Ki67 and cleaved caspase-3 immunohistochemical staining in colon cancer tissues.
A) Ki67 tumor staining with low expression; B) Ki67tumor staining with high expression; C) Tumor showing absence of cleaved Caspase-3 tumor cell expression and; D) Tumor showing presence of cleaved caspase-3 tumor cell expression, as indicated by the arrows. All magnifications x200.

Determination of microsatellite stability status

DNA was extracted from 2mm tumor-cores. Paraffin was dissolved in xylene, tissue was rehydrated in ethanol (100%/70%) and dried for 10 minutes at 37°C. Nucleospin 96 Tissue kit (Machery-Nagel, Düren, Germany) was used for DNA extraction according to the manufacturer's protocol.

Microsatellite stability status was tested using the MSI Analysis System Version 1.2 (Promega, Mannheim, Germany) and interpreted by an experienced pathologist, as described previously [23].

Statistical Analysis

Statistical analyses were performed using the statistical package SPSS (version 17.0 for Windows; SPSS, inc). The Student's T-test and the Chi-squared test were used to evaluate associations between Ki67 or cleaved caspase-3 and various clinico-pathological parameters. The Overall Survival (OS) was defined as time between primary tumor resection and time of death and Disease Free Survival (DFS) as time between primary tumor resection and time of death or relapse of disease, whichever came first. The Kaplan-Meier method was used for calculation of survival probabilities and the Log-rank test for comparison of survival curves. Cox regression was used for univariate and multivariable analysis for OS and DFS. Significant variables (in univariate analysis) were included in multivariable analysis. For all tests, a p-value <0.05 was considered to be statistical significant.

RESULTS

Patient characteristics, and cleaved caspase-3 and Ki67 expression levels

The study cohort consisted of 285 patients. In 41 cases for Ki67, and 38 for cleaved caspase-3 the results of the IHC could not be analyzed due to loss of the tumor material during IHC or due to staining artifacts. Representative images of the biomarkers and their staining categories are shown in Figure 1. The mean percentage of tumor cells expressing Ki67 in the tumor tissue cores was 29.2% with a median of 27.5%. For analysis we used the median as cutoff based on skewness of the data distribution. This resulted in 121 patients (49.6%) with tumors showing low expression level (below median) of Ki67 and ('low' tumor cell proliferation level) and 123 patients (50.4%) with high expression level (above median) of Ki67 ('high' tumor cell proliferation level). In 85 (34.4%) patients the tumor tissue cores showed no staining of cleaved caspase-3 and therefore no apoptotic activity of tumor cells. The remaining 65.6% of the samples showed positive staining and thus ongoing tumor cell apoptosis.

The clinico-pathological characteristics of the patient cohort and their relation to expression levels of the biomarkers are listed in Table I. Interestingly, tumor location

Table I: Patient Characteristics of the Total Colon Cancer Cohort and Stratified for Ki67 and cCaspase-3 expression

	Total Population (N=285)	Ki67 Absence N=121	Ki67 Presence N=123	cCaspase-3 Absence N=85	cCaspase-3 Presence N=162
Gender					
Male	137 (48.1%)	55 (45.5%)	65 (52.8%)	42 (49.4%)	80 (49.4%)
Female	148 (51.9%)	66 (54.5%)	58 (47.2%)	43(50.6%)	82 (50.6%)
Age (average)	65.7 (±13.3 SD)	67.3 (±11.6 SD)	64.2 (±13.6 SD)	66.5 (±12.6 SD)	65.6 (±12.9 SD)
TNM stage					
I	44 (15.4%)	14 (11.6%)	20 (16.3%)	16(18.8%)	18 (11.1%)
II	114 (40.0%)	43 (35.5%)	53 (43.1%)	31 (36.5%)	68 (42.0%)
III	74 (26.0%)	36 (29.8%)	30 (24.4%)	19 (22.4%)	47 (29.0%)
IV	48 (16.8%)	27 (22.3%)	19 (15.4%)	17 (20.0%)	29 (17.9%)
Unknown	5 (1.8%)	1 (0.8%)	1 (0.8%)	2 (2.4%)	0 (0.0%)
Grade			*p=0.004*		*p=0.017*
Moderate	145 (50.9%)	61 (50.4%)	63 (51.2%)	35(41.2%)	89 (54.9%)
Poor	23 (8.1%)	5 (4.1%)	16 (13.0%)	4 (4.7%)	18(11.1%)
Good	58 (20.4%)	32 (26.4%)	16 (13.0%)	22(25.9%)	26 (16.0%)
Unknown	59 (20.7%)	23 (19.0%)	28 (22.8%)	24 (28.2%)	29(17.9%)
MS Status					*p=0.004*
MSS	168 (58.9%)	77 (63.6%)	87 (70.7%)	61 (71.8%)	103 (63.6%)
MSI	30 (10.5%)	12 (9.9%)	14 (11.4%)	2 (2.4%)	27 (16.7%)
Unknown	87 (30.5%)	32 (26.4%)	22 (17.9%)	22 (25.9)	32 (19.8%)
Location			*p=0.023*		*p=0.011*
Right	110 (38.6%)	37 (30.6%)	53 (43.1%)	27 (31.8%)	64(39.5%)
Left	153 (53.7%)	78 (64.5%)	60 (48.8%)	57 (67.1%)	83(51,2%)
Unknown	22 (7.7%)	6 (5.0%)	10 (8.1%)	1 (1.2%)	15 (9.3%)

This table describes the baseline characteristics of the entire cohort of 285 patients in the first column. The Ki67 immunohistochemistry results could be analyzed in 244 cases and Ki67 expression (above the median of 27.5% expression level) was found to be present in 123 and absent in 121 patients. The second and third columns describe the relation of either Ki67 absence or presence in the tumor resection specimens to clinico-pathological parameters. The cleaved caspase 3 results were available for analysis in 247 patients. In this population 85 tumor samples showed no presence of cleaved caspase 3, expression was present in 162 tumor samples of patients. The fourth and fifth column describe the relation of either cleaved caspase 3 absence or presence to clinico-pathological parameters. Only significant ($p<0.05$) differences between marker expression as proven by χ^2 tests are displayed. Abbreviations: MS Status; Microsatellite Status, cCaspase3; cleaved caspase 3.

was significantly related to both cleaved caspase-3 expression level and Ki67 expression level. Microsatellite instability also showed statistical significance, but was only significantly related to cleaved caspase-3 expression and not to Ki67 expression. In the tumor samples without cleaved caspase-3 expression, 2.4% of the cases showed microsatellite instability *vs.* 16.7% in the tumors with expression of cleaved caspase-3 (*p*-value 0.004). Additional analysis showed in our patient cohort a strong, significant correlation between tumor location and microsatellite stability status with significantly more microsatellite instable tumors (MSI) located on the right side of the colon and the majority of the microsatellite stable (MSS) tumors located on the left side of the colon (70%), whereas this was only 8% in microsatellite instable tumors (MSI) (*p*=<0.001).

Relation of single marker expression with patient outcome

The level of tumor cell proliferation based on Ki67 expression level was significantly related to OS and DFS: high tumor expression level correlated significantly to a better patient OS and DFS (OS, Logrank *p*-value 0.002; DFS, Logrank *p*-value 0.003) (Figure 2). Tumor cell apoptotic level, as represented by cleaved caspase-3 expression, was not related to either OS or DFS (OS, Logrank *p*-value 0.83; DFS, Logrank *p*-value 0.73).

Combined analysis of tumor cell apoptosis and proliferation in relation to patient outcome

To analyze the effect of the balance between apoptosis and proliferation levels in the tumor resection specimens on patient outcome, the results of the Ki67 expression analysis were combined with those of the cleaved caspase-3 expression analysis in a combined apoptosis-proliferation (CAP) parameter (Table II). The CAP parameter was not significantly related to TNM stage (*p*-value 0.211), but was significantly related to the tumor microsatellite status (*p*-value 0.008). Tumors of both the CAP -+ (Ki67 below median, presence of cleaved caspase-3) and ++ (Ki67 above median, presence of cleaved caspase-3) patients showed significantly more often microsatellite stability compared to the CAP +- (Ki67 above median, absence of cleaved caspase-3) and CAP — (Ki67 below median, absence of cleaved caspase-3) patients (*p*-value 0.03). Patients with a CAP ++ tumor showed the best survival outcomes with respect to OS and DFS (Figure 3). In the entire cohort patients with a CAP -+ tumor had the worst outcome perspectives. Because tumor microsatellite status was significantly related to the presence of cleaved caspase-3, the next step would be to perform the survival analysis with the CAP parameters stratified for tumor microsatellite status. Unfortunately the number of MSI tumors that was successfully determined was too small to perform this analysis specifically for MSI within this population. We therefore used tumor location, which we previously showed to be highly correlated to tumor microsatellite status, as a surrogate marker in this analysis (Figure 4). These Kaplan Meier curves showed in left-sided tumors

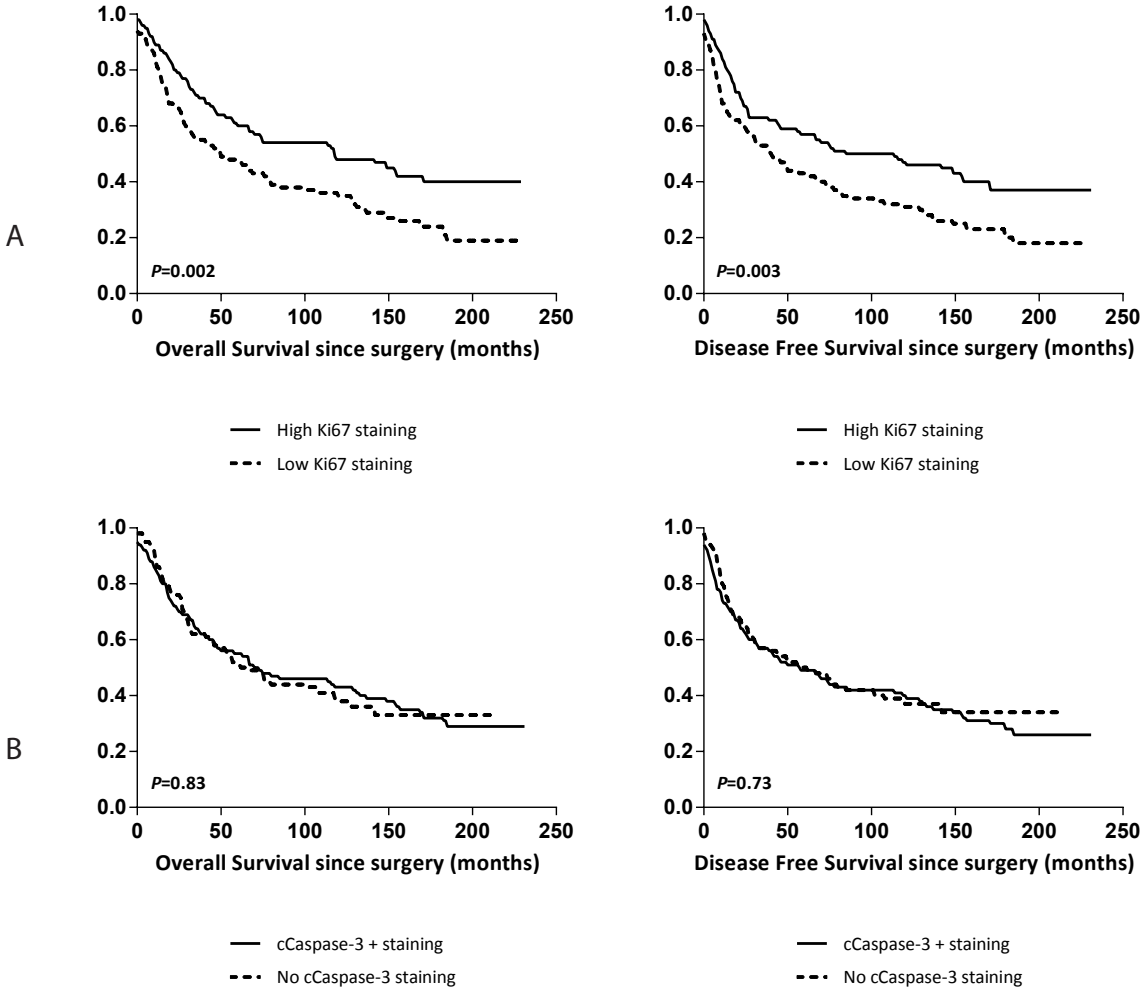


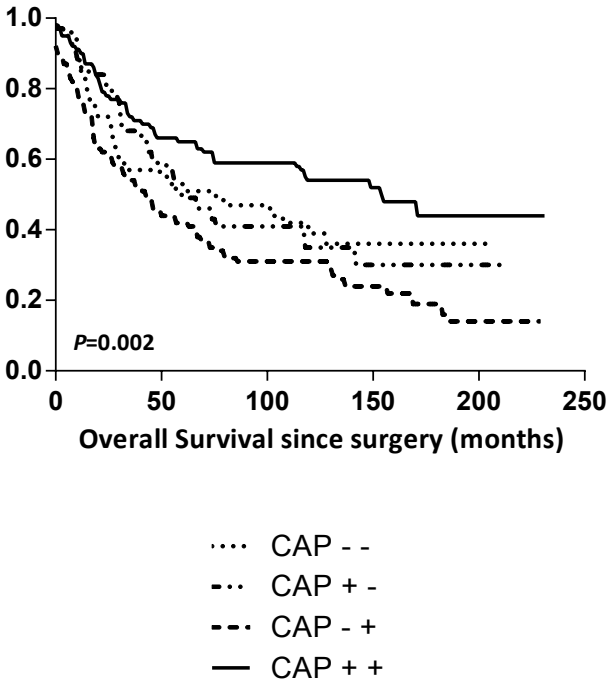
Figure 2: Survival curves stratified for Ki67 and cleaved caspase-3 tumor expression in colon cancer. A) Kaplan Meier curves for OS and DFS in the study population of 285 colon cancer patients stratified for Ki67 tumor expression. B) Kaplan Meier curve OS and DFS in the study population of 285 colon cancer patients stratified for cleaved caspase-3 expression in their tumor sections. Abbreviations: cCaspase3; cleaved caspase-3.

Table II: Description of the CAP (Combined Apoptosis and Proliferation) parameter.

CAP ++	Ki67 expression above the median and presence of cleaved caspase-3 IHC
CAP +-	Ki67 expression above the median and no presence of cleaved caspase-3 IHC
CAP -+	Ki67 expression below the median and presence of cleaved caspase-3 IHC
CAP —	Ki67 expression below the median and no presence of cleaved caspase-3 IHC

This table provides the definitions of the CAP parameter. This parameter resulted from data combination on the tumor cell apoptotic level based on the cleaved caspase-3 immunohistochemistry results with the data on the tumor cell proliferation level based on the Ki67 expression levels as determined with IHC. Abbreviations: IHC; immunohistochemistry.

A



B

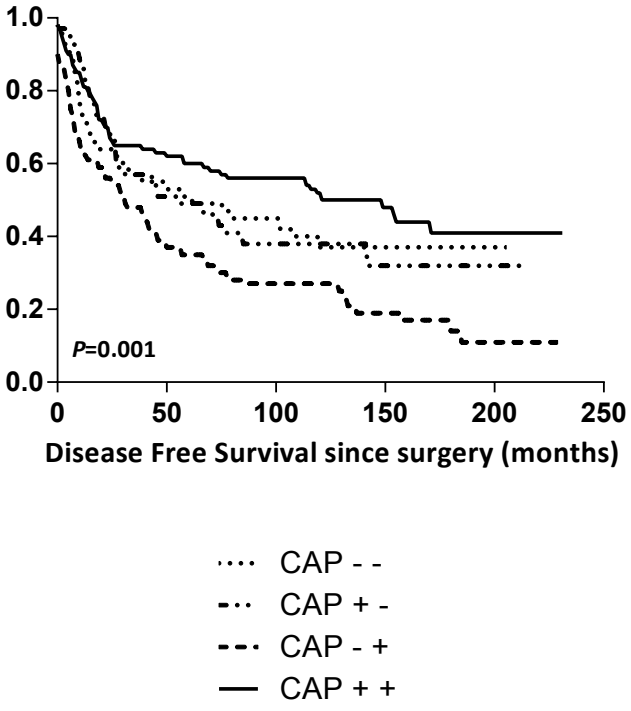


Figure 3: Survival curves stratified for combined tumor apoptosis-proliferation (CAP) expression in colon cancer.

Kaplan Meier curves for OS and DFS in the study population of 285 colon cancer patients stratified for combined tumor apoptosis-proliferation (CAP) expression. This parameter is described in detail in Table II and in the *Results* section.

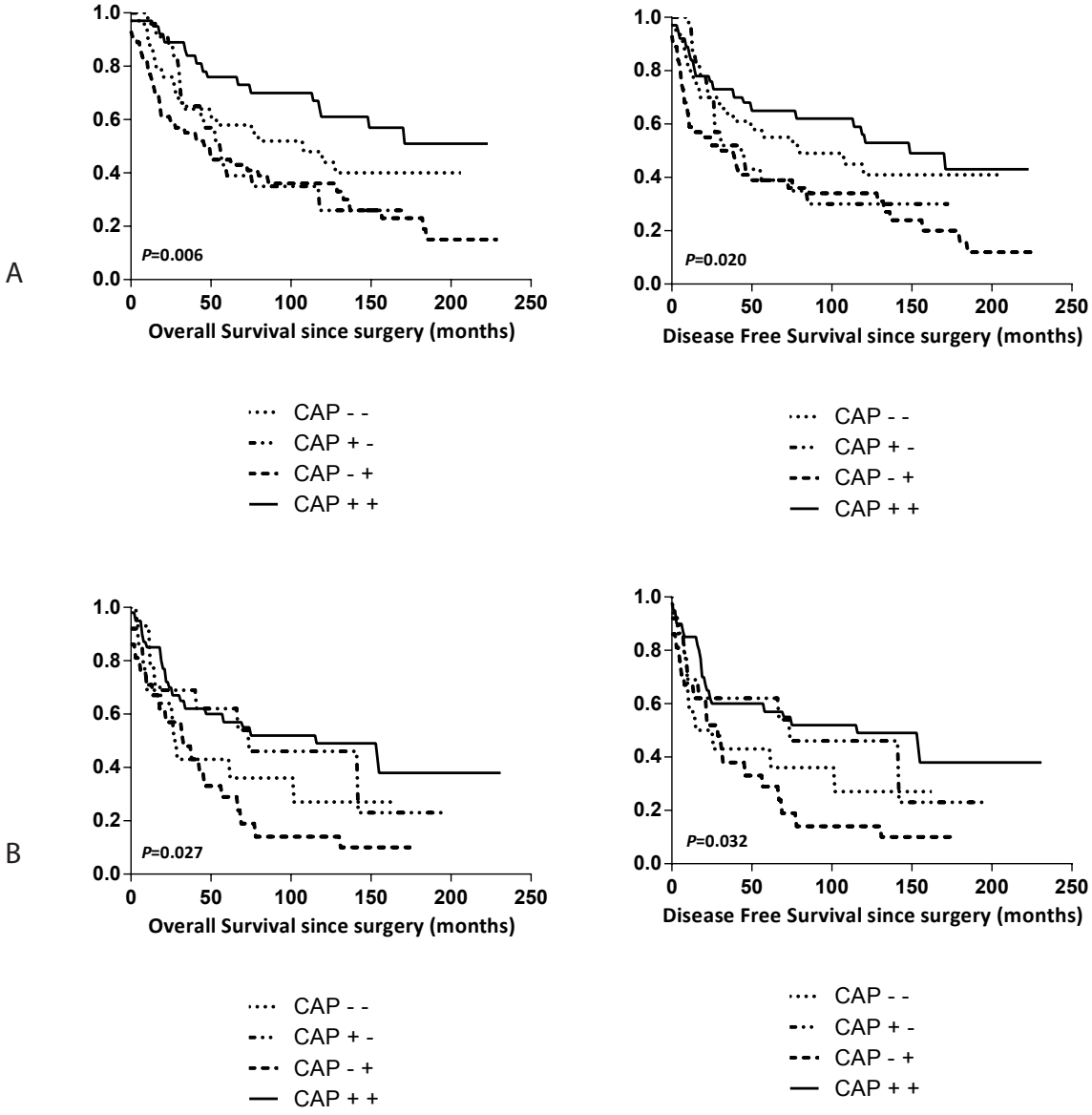


Figure 4: Survival curves stratified for combined tumor apoptosis-proliferation expression and for location of the colon tumor.

A) Kaplan Meier curves for OS and DFS stratified for the CAP parameter in patients with left sided colon tumors (originating distal to the splenic flexure). B) Kaplan Meier curves for OS and DFS in patients with right-sided tumors (originating proximal to the splenic flexure). The CAP parameter is described in detail in Table II and in the *Results* section.

comparable curves to those presented in Figure 3 of the total cohort, but the course of the curves changed in right-sided tumors. The CAP ++ and the CAP - - population within the cohort of left-sided tumors had the best outcome perspectives as opposed to the CAP -+ and +- population that had similar but worse outcome perspectives. The CAP + - actually had, within this left-sided cohort, the worst outcome perspectives (DFS *p*-value 0.02). In right-sided tumors, the CAP ++ and CAP +- population had the best outcome perspectives as opposed to the CAP — and CAP -+ population that had worse

outcome perspectives. We conclude based on these results that combined analysis of apoptosis and proliferation as described with the CAP parameter is related to survival in stage I-IV colon cancer patients. The impact of this parameter on patient outcome, however, varies with tumor location and therefore highly likely with tumor microsatellite status.

Univariate and multivariable analysis

Both for OS and DFS a multivariable analysis was performed including the variables; sex, age at time of operation, TNM stage, tumor grade, administration of adjuvant therapy, microsatellite status, tumor location and the CAP parameter. Age and TNM stage were found to be independent predictors of OS and DFS (Table III & IV). To test whether the effect of the CAP parameter on outcome differed between patients with left- and right-sided tumors, an interaction term was implemented that was borderline significant (*p*-value 0.06). Although the CAP parameter was not significantly related to TNM stage, stratified analyses for TNM stage showed that the effect of CAP on outcome only remained significant in the stage II patient population. Therefore analysis was again performed with an interaction term, and again this term was borderline significant (*p*-value 0.05).

Table III: Univariate and multivariable analyses of Overall Survival (OS)

	Univariate analysis			Multivariable analysis		
	HR	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
TNM			<0.001			<0.001
1	1			1		
2	1.57	0.9-2.6		2.01	1.1-3.7	
3	2.17	1.3-3.6		2.61	1.4-4.8	
4	6.27	3.6-10.7		7.67	4.1-14.3	
Age	1.04	1.026-1.053	<0.0001	1.054	1.037-1.071	<0.001
CAP			0.006			0.28
—	1.00			1		
-+	1.42	0.9-2.4		1.10	0.6-1.9	
+-	0.71	0.5-1.0		1.27	0.8-2.0	
++	0.52	0.3-0.9		0.85	0.5-1.4	

This table provides the data for the univariate en multivariable analysis of OS. The univariate analysis included sex, age, tumor grade, adjuvant therapy administration, microsatellite status, TNM stage, the CAP parameter, microsatellite status and tumor location. The CAP parameter, age and TNM stage were all significant predictors of OS in univariate analysis. In multivariable analysis only TNM stage and the patient age at time of surgery retained significance. The CAP parameter is therefore not an independent predictor of overall survival in stage I-IV in this cohort of colon cancer patients.

Table IV: Univariate and multivariable analyses of Disease Free Survival (DFS)

	Univariate analysis			Multivariable analysis		
	HR	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
TNM			<0.001			<0.001
1	1			1		
2	1.56	0.9-2.5		1.94	1.1-3.5	
3	2.25	1.4-3.7		2.52	1.4-4.6	
4	6.14	3.6-10.4		7.17	3.9-13.1	
Age	1.032	1.019-1.045	<0.001	1.043	1.028-1.059	<0.001
CAP			0.02			0.235
—	1			1		
+-	1.04	0.6-1.8		1.08	0.6-1.8	
-+	1.60	1.0-2.5		1.37	0.9-2.1	
++	0.75	0.5-1.2		0,91	0.6-1.5	

This table provides the data for the univariate en multivariable analysis of DFS The univariate analysis included sex, age, tumor grade, adjuvant therapy administration, microsatellite status, TNM stage, the CAP parameter, microsatellite status and tumor location. The CAP parameter, age and TNM stage were all significant predictors of DFS in univariate analysis. In multivariable analysis only TNM stage and the patient age at time of surgery retained significance. The CAP parameter is therefore not an independent predictor of disease free survival in this cohort of stage I-IV colon cancer patients.

Based on these results we conclude that although the CAP parameter is not a statistically independent prognostic indicator of survival in the total patient cohort, the CAP parameter, which is influenced by location, microsatellite stability status and TNM stage, does behold prognostic significance in certain subsets of patients populations such as in stage II, MSS patients.

DISCUSSION

Our study shows that a combined parameter, CAP, describing the level of tumor cell proliferation and apoptosis is significantly related to patient outcome in a stage I-IV colon cancer patient cohort with respect to DFS and OS. Although counterintuitively, patients with CAP ++ tumor, showing high levels of both proliferation and apoptosis, showed the best clinical outcome perspectives. The effect of the CAP parameter, however, varied with TNM stage and tumor location and was significantly related to tumor location and tumor microsatellite status. These results confirm our hypothesis that clinical outcome is dependent on both tumor cell proliferation and apoptosis.

The processes of both tumor cell proliferation and apoptosis both have been extensively studied with varying results in many types of cancer. In general, high tumor cell

proliferation levels were associated with aggressive tumor development and progression [20;24]. However, other studies reported on an inverse association between tumor cell proliferation level and clinical outcome [8;17;22;25]. These latter results are in line with what we have found: a better outcome perspective in colon cancer patients with high levels of tumor cell proliferation. In this study we were not able to establish a relationship between the level of apoptosis as a single marker and patient outcome in colon cancer patients. Although there are studies that describe a link between tumor cell apoptosis and clinical outcome, for example Jonges *et al.* who described cleaved caspase-3 expression as a prognostic marker in colon cancer patients [19], the majority of the studies have presented us with more ambiguous results [1;15;16;26;27].

The contradicting results derived from studies reporting on either proliferation or apoptosis in colon cancer strengthened our hypothesis that a balance between both these processes determines patient's clinical outcome. Michael-Robinson *et al.* previously reported on a cohort of 100 colorectal cancer patients in which they determined an Apoptotic Index: Proliferation Index (AI:PI) ratio [25]. This AI:PI ratio was based on M30 IHC for the apoptosis level and Ki67 IHC for the proliferation level. They were able to determine a relationship between the proliferation index and outcome comparable to our results: they also related their AI:PI index significantly to patient outcome. In previous studies the use of the apoptotic index has been criticized as researches found the use of the parameter to be accompanied with high amounts of interobserver variability [28]. Therefore we didn't use a continuous variable based on counted percentages, but developed a more descriptive parameter, the CAP, to determine the combined effect of apoptosis and proliferation within our patients tumor samples. The differences in outcome parameters and also patient selection make it difficult to perform a one-to-one correlation of the results of Michael-Robinson *et al.* and our results. Their conclusions though do affirm our hypothesis. Interestingly, the survival difference they found between a high and low AI:PI index was similar in both MSS and MSI patients. Our results showed that the effect of the CAP parameter differed between tumors emerging from colon proximal and colon distal to the splenic flexure. In the left-sided cohort the patients with CAP — and ++ tumors performed better with respect to outcome. In the right-sided cohort the CAP +- performed significantly better than the CAP — cohort. This is comparable to what we have previously found and described by Jonges *et al.* [19]. The effect of apoptosis on patient outcome is related to tumor location. Based on our results we hypothesize that it is either tumor microsatellite status as suggested by both Jonges and Michael-Robison, or tumor location which might influence the balance between tumor cell proliferation and apoptosis and therefore patient outcome [19;25]. The concept of the effect of tumor location is in accordance with what has recently been described by The Cancer Genome Atlas Network in their publication in Nature in 2012, who tested the hypothesis that differences between tumors originating from the left

or the right side of the colon is not based on their microsatellite status but it might be caused by the different embryonic origins of the right- and left-sided colon [29].

It is not unlikely that the tumor microsatellite status influences the balance between tumor cell proliferation and apoptosis. Microsatellite instable tumors are known to have high levels of proliferation and tend to accumulate gene mutations leading to increased production of abnormal peptides [30;31]. This phenotype has been hypothesized to cause an immune reaction resulting in higher levels of apoptosis, eventually resulting in better patient outcome [32].

In conclusion, the CAP variable described in this study reflects the balance between the apoptosis and proliferation in colon cancer tissue and showed to be related to patient outcome. These results confirm our hypothesis that apoptosis and proliferation together determine patient outcome in colon cancer and this relation is influenced by tumor location and/ or by tumor microsatellite instability. This was shown by the different effects of the CAP parameter on patient outcome in the left and right-sided colon cancer patients cohorts and the statically significant relation of the level of apoptosis with tumor microsatellite status, also described in previous studies [19;25] . Important steps have been taken towards the implementation of a CAP like parameter into clinical practice, such as the development of the CDK1 SA (Cyclin Dependent Kinase 1 Specific Activity Assay), a biochemical assay that can replace the Ki67 IHC, and the improvement of the existing biochemical assays to measure cleaved caspase-3 activity for easy clinical use [23]. Further studies should focus on the design of clinical tests combining both proliferation-based markers and apoptosis-based markers into one analysis to assure clinical applicability.

REFERENCE LIST

(1) Bedi A, Pasricha PJ, Akhtar AJ et al. Inhibition of apoptosis during development of colorectal cancer. *Cancer Res* 1995;55:1811-1816.

(2) Ramachandran A, Madesh M, Balasubramanian KA. Apoptosis in the intestinal epithelium: its relevance in normal and pathophysiological conditions. *J Gastroenterol Hepatol* 2000;15:109-120.

(3) Takano Y, Saegusa M, Ikenaga M, Mitomi H, Okayasu I. Apoptosis of colon cancer: comparison with Ki-67 proliferative activity and expression of p53. *J Cancer Res Clin Oncol* 1996;122:166-170.

(4) Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57-70.

(5) Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646-674.

(6) Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. *Nature* 2001;411:342-348.

(7) Flohil CC, Janssen PA, Bosman FT. Expression of Bcl-2 protein in hyperplastic polyps, adenomas, and carcinomas of the colon. *J Pathol* 1996;178:393-397.

(8) Garrity MM, Burgart LJ, Mahoney MR et al. Prognostic value of proliferation, apoptosis, defective DNA mismatch repair, and p53 overexpression in patients with resected Dukes' B2 or C colon cancer: a North Central Cancer Treatment Group Study. *J Clin Oncol* 2004;22:1572-1582.

(9) Moss SF, Liu TC, Petrotos A, Hsu TM, Gold LI, Holt PR. Inward growth of colonic adenomatous polyps. *Gastroenterology* 1996;111:1425-1432.

(10) Sinicrope FA, Roddey G, McDonnell TJ, Shen Y, Cleary KR, Stephens LC. Increased apoptosis accompanies neoplastic development in the human colorectum. *Clin Cancer Res* 1996;2:1999-2006.

(11) Sinicrope FA, Hart J, Hsu HA, Lemoine M, Michelassi F, Stephens LC. Apoptotic and mitotic indices predict survival rates in lymph node-negative colon carcinomas. *Clin Cancer Res* 1999;5:1793-1804.

(12) Sugao Y, Koji T, Yao T, Ueki T, Tsuneyoshi M. The Incidence of Apoptosis During Colorectal Tumorigenesis. *Int J Surg Pathol* 2000;8:123-132.

(13) Brown JM, Attardi LD. The role of apoptosis in cancer development and treatment response. *Nat Rev Cancer* 2005;5:231-237.

(14) Scopa CD, Tsamandas AC, Zolota V, Kalofonos HP, Batistatou A, Vagianos C. Potential role of bcl-2 and ki-67 expression and apoptosis in colorectal carcinoma: a clinicopathologic study. *Dig Dis Sci* 2003;48:1990-1997.

(15) de Bruin EC, van de Velde CJ, van de Pas S et al. Prognostic value of apoptosis in rectal cancer patients of the dutch total mesorectal excision trial: radiotherapy is redundant in intrinsically high-apoptotic tumors. *Clin Cancer Res* 2006;12:6432-6436.

(16) de Heer P, de Bruin EC, Klein-Kranenbarg E et al. Caspase-3 activity predicts local recurrence in rectal cancer. *Clin Cancer Res* 2007;13:5810-5815.

(17) Fluge O, Gravdal K, Carlsen E et al. Expression of EZH2 and Ki-67 in colorectal cancer and associations with treatment response and prognosis. *Br J Cancer* 2009;101:1282-1289.

(18) Guastadisegni C, Colafranceschi M, Ottini L, Dogliotti E. Microsatellite instability as a marker of prognosis and response to therapy: a meta-analysis of colorectal cancer survival data. *Eur J Cancer* 2010;46:2788-2798.

(19) Jonges LE, Nagelkerke JF, Ensink NG et al. Caspase-3 activity as a prognostic factor in colorectal carcinoma. *Lab Invest* 2001;81:681-688.

(20) Kimura T, Tanaka S, Haruma K et al. Clinical significance of MUC1 and E-cadherin expression, cellular proliferation, and angiogenesis at the deepest invasive portion of colorectal cancer. *Int J Oncol* 2000;16:55-64.

(21) Pollack A, DeSilvio M, Khor LY et al. Ki-67 staining is a strong predictor of distant metastasis and mortality for men with prostate cancer treated with radiotherapy plus androgen deprivation: Radiation Therapy Oncology Group Trial 92-02. *J Clin Oncol* 2004;22:2133-2140.

(22) Salminen E, Palmu S, Vahlberg T, Roberts PJ, Soderstrom KO. Increased proliferation activity measured by immunoreactive Ki67 is associated with survival improvement in rectal/recto sigmoid cancer. *World J Gastroenterol* 2005;11:3245-3249.

(23) Zeestraten EC, Maak M, Shibayama M et al. Specific activity of cyclin-dependent kinase I is a new potential predictor of tumour recurrence in stage II colon cancer. *Br J Cancer* 2012;106:133-140.

(24) Menezes HL, Juca MJ, Gomes EG, Nunes BL, Costa HO, Matos D. Analysis of the immunohistochemical expressions of p53, bcl-2 and Ki-67 in colorectal adenocarcinoma and their correlations with the prognostic factors. *Arq Gastroenterol* 2010;47:141-147.

(25) Michael-Robinson JM, Reid LE, Purdie DM et al. Proliferation, apoptosis, and survival in high-level microsatellite instability sporadic colorectal cancer. *Clin Cancer Res* 2001;7:2347-2356.

(26) Baretton GB, Diebold J, Christoforis G et al. Apoptosis and immunohistochemical bcl-2 expression in colorectal adenomas and carcinomas. Aspects of carcinogenesis and prognostic significance. *Cancer* 1996;77:255-264.

(27) Kawasaki H, Altieri DC, Lu CD, Toyoda M, Tenjo T, Tanigawa N. Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. *Cancer Res* 1998;58:5071-5074.

(28) Soini Y, Paakko P, Lehto VP. Histopathological evaluation of apoptosis in cancer. *Am J Pathol* 1998; 153:1041-1053.

(29) Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 2012;487: 330-337.

(30) Kim GP, Colangelo LH, Wieand HS et al. Prognostic and predictive roles of high-degree microsatellite instability in colon cancer: a National Cancer Institute-National Surgical Adjuvant Breast and Bowel Project Collaborative Study. *J Clin Oncol* 2007;25:767-772.

(31) Okon K, Demczuk S, Klimkowska A et al. Correlation of microsatellite status, proliferation, apoptotic and selected immunohistochemical markers in colorectal carcinoma studied with tissue microarray. *Pol J Pathol* 2006;57:105-111.

(32) Dolcetti R, Viel A, Doglioni C et al. High prevalence of activated intraepithelial cytotoxic T lymphocytes and increased neoplastic cell apoptosis in colorectal carcinomas with microsatellite instability. *Am J Pathol* 1999;154:1805-1813.

CHAPTER 5

Validation of the 12-gene Colon Cancer Recurrence Score as a predictor of recurrence risk in stage II and III rectal cancer patients

Marlies S. Reimers, Peter J.K. Kuppen, Mark Lee, Margarita Lopatin, Haluk Tezcan, Hein Putter, Kim Clark-Langone, Gerrit Jan Liefers, Steve Shak, Cornelis J.H. van de Velde

Journal of the National Cancer Institute 2014 Sep 26; 106 (11)

ABSTRACT

Background

The 12-gene Recurrence Score assay is a validated predictor of recurrence risk in stage II and III colon cancer patients. We conducted a prospectively designed study to validate this assay for prediction of recurrence risk in stage II and III rectal cancer patients from the Dutch Total Mesorectal Excision (TME) trial.

Methods

RNA was extracted from fixed paraffin-embedded primary rectal tumor tissue from stage II and III patients randomized to TME surgery alone, without (neo)adjuvant treatment. Recurrence Score was assessed by quantitative RT-PCR using previously validated colon cancer genes and algorithm. Data were analysed by Cox proportional hazards regression adjusting for stage and resection margin status.

Results

Recurrence Score predicted risk of recurrence (p=0.011), risk of distant recurrence (p=0.030), and rectal cancer-specific survival (p=0.007). The effect of Recurrence Score was most prominent in stage II patients and attenuated with more advanced stage (interaction $p \leq 0.007$ for each endpoint). In stage II, 5-year cumulative incidence of recurrence ranged from 11% in the pre-defined low Recurrence Score group (48% of pts) to 43% in the high Recurrence Score group (23% of pts).

Conclusions

The 12-gene Recurrence Score is a predictor of recurrence risk and cancer specific survival in rectal cancer patients treated with surgery alone, suggesting a similar underlying biology in colon and rectal cancers.

INTRODUCTION

Before the introduction of the total mesorectal excision (TME) technique, which resulted in a substantial decrease in local recurrences and improved survival, the 5-year local recurrence rate of rectal cancer with conventional surgery was over 20% [1]. Between 1996-1999, the Dutch TME trial investigated the effect of short-term preoperative radiotherapy in combination with TME surgery compared to TME surgery alone in 1861 rectal cancer patients [2]. Five and ten year results of this trial showed improved local recurrence rates in patients treated with preoperative radiotherapy and TME [3-5]. However, no significant effect was seen on distant recurrence and overall survival (OS) [5].

While TME surgery and preoperative therapy have reduced local recurrence, the role of adjuvant chemotherapy in rectal cancer in reducing distant recurrence rates and improving OS remains controversial. In a systematic review and meta-analysis of 21 randomized clinical trials, the use of 5-fluorouracil (5-FU) based adjuvant chemotherapy for rectal cancer patients who received no preoperative therapy was found to improve both OS and disease-free survival (DFS) [6]. However, for rectal cancer patients receiving preoperative chemo- or radiotherapy, most trials did not show a survival benefit for adjuvant chemotherapy [7-10]. Current clinical and pathologic features in rectal cancer are not able to adequately characterize recurrence risk. As such, aggressive approaches combining preoperative chemoradiation, TME surgery, and in some countries, postoperative adjuvant chemotherapy continue to be used in stage III and many stage II rectal cancers, with attendant clinical toxicity, patient burden, and financial cost. There is thus a strong need for new clinical tools which more accurately identify patients with low and high-risk of recurrence; especially for stage II patients, a more individualized approach to balancing risk of recurrence, modest treatment benefit, and therapy-related toxicities should improve treatment decision-making.

The 12-gene Recurrence Score assay (Genomic Health, Redwood City, CA, USA) was developed by using tumor gene expression data from 1851 patients with resected colon cancer from four independent clinical trials [11]. This 12-gene assay, measuring expression of 12 genes (seven recurrence and five reference genes) in fixed, paraffin-embedded (FPE) primary colon tumor tissue, was validated as a predictor of recurrence risk in stage II and III colon cancer patients from QUASAR, Cancer and Leukemia Group B (CALGB) 9581, and National Surgical Adjuvant Breast and Bowel Project (NSABP) C-07 trials [12-14], providing risk discrimination beyond conventional clinical and pathologic factors.

The purpose of this prospectively-designed study was to validate the 12- gene Recurrence Score assay in stage II and III rectal cancer for recurrence risk prediction in patients from the TME alone arm of the Dutch TME trial who received no pre- and postoperative therapy.

METHODS

Patients and Tissue Specimens

Stage II and III rectal cancer patients enrolled in the Dutch TME trial, randomized to surgery alone, underwent radical resection (i.e. R0-R1), were treated per TME trial protocol and had FPE tumor tissue were eligible for the study [3]. Informed consent was obtained from all patients enrolled in the TME trial. The study was approved by the Medical Ethical Committee of the Leiden University Medical Center. Per TME protocol, patients with tumor spillage during operation or tumor-positive resection margin were allowed to receive radiotherapy. Follow-up assessments involved clinical evaluation every three months during the first year after surgery and yearly for at least two more years, including liver imaging and endoscopy. Additionally, chest X-ray/CT, CEA determination and endo-ultrasound were performed on indication.

Pathology and Gene expression

Pathologic T-stage, number of nodes examined and involved by carcinoma, resection margin status, distance from anal verge, and local grade assessments were obtained from the TME clinical database. Positive resection margin (RM) was defined as positive circumferential, distal, proximal, or nodal margin, or presence of the tumor ≤ 1mm from any of these margins. In addition, tumor type and grade were centrally assessed [15] according to WHO guidelines [16] by an academic surgical pathologist specialized in gastrointestinal pathology.

RNA was extracted from six 5-µm sections, quantified by RiboGreen (Invitrogen, Carlsbad, CA) and analysed by reverse transcriptase quantitative polymerase chain reaction using a standardized, analytically validated process [17]. The 12-gene Recurrence Score results were calculated using prespecified genes and algorithm, as previously validated in QUASAR, CALGB 9581, and NSABP C-07 [12-14]. Prespecified cut points were used to define low, intermediate, and high Recurrence Score groups (i.e., RS<30, 30 to 40, and ≥ 41 respectively) [12].

All centrally-performed pathology and laboratory procedures were prespecified and conducted without knowledge of patient clinical characteristics or outcomes.

Statistical Methods

The prespecified primary study endpoint was recurrence-free interval (RFI), defined as time from surgery to first rectal cancer recurrence (local or distant) or death with a documented recurrence at time of death. Local recurrence was defined as tumor within the lesser pelvis or perineal wound and distant recurrence as tumor in any other area including at the colostomy site or in the inguinal region [3]. Deaths without evidence of recurrence and losses to follow-up were censored. Second primary cancers were

ignored. RFI was chosen as primary endpoint, as opposed to time to local recurrence in the parent TME trial, because gene expression was expected to be associated with any recurrence of the primary tumor and most recurrences in rectal cancer are distant.

Secondary endpoints included distant RFI (DRFI), where local recurrences were neither censored nor considered as events, rectal cancer-specific survival (RCSS), where death is either preceded by rectal cancer recurrence or occurs with documented recurrence, DFS, and OS.

The primary analysis model used Cox proportional hazards (PH) regression to evaluate the association between continuous Recurrence Score results and outcome, adjusted for stage (II, IIIA/B, IIIC corresponding to 0, 1-3 and 4+ positive nodes, respectively) and RM status (RM-negative, RM-positive treated with surgery alone, and RM-positive treated with surgery followed by radiotherapy). A two-sided p-value < 0.05, based on a likelihood ratio test, was considered significant. The hazard ratio for Recurrence Score was reported for an increase of 25 units, consistent with previous studies. Proportional hazards were assessed by examining the relationship between scaled Schoenfeld residuals and time. Non-linearity was assessed by a likelihood ratio test for squared and cubic terms for Recurrence Score results. Stage-specific additive splines that were constrained to be linear in the tails [18] were used to model non-linear effects of the continuous Recurrence Score. Contribution of Recurrence Score beyond prespecified pathologic covariates was evaluated using multivariable Cox PH models. The relationship between Recurrence Score groups and RFI, DRFI and RCCS was characterized by cumulative incidence estimates and Aalen's estimates of variance accounting for death without evidence of recurrence and death due to cancers other than rectal cancer as competing risks [19]. Additionally, Kaplan-Meier methods were used. Relative utility curves and a test tradeoff were computed [20;21]. Analyses used IBM SPSS Statistics 20, R version 2.14.0 (cmprsk and mstate packages) and SAS version 9.2.

RESULTS

Patient characteristics

Tumor tissue was available for 308 (59%) of 518 eligible stage II and III patients in the TME trial who were randomized to surgery alone. Following prespecified procedures for pathology and laboratory processing, 11 (3.6%) patients were excluded, primarily for insufficient tumor tissue (Figure 1). The final evaluable data set contained 297 patients with 128 (43%) recurrences, including 50 (17%) local and 112 (38%) distant recurrences (34 patients had both local and distant recurrence). Recurrences were observed in 34 (26%) of 130 stage II patients, 57 (52%) of 110 stage IIIA/B patients and 37 (65%) of 57

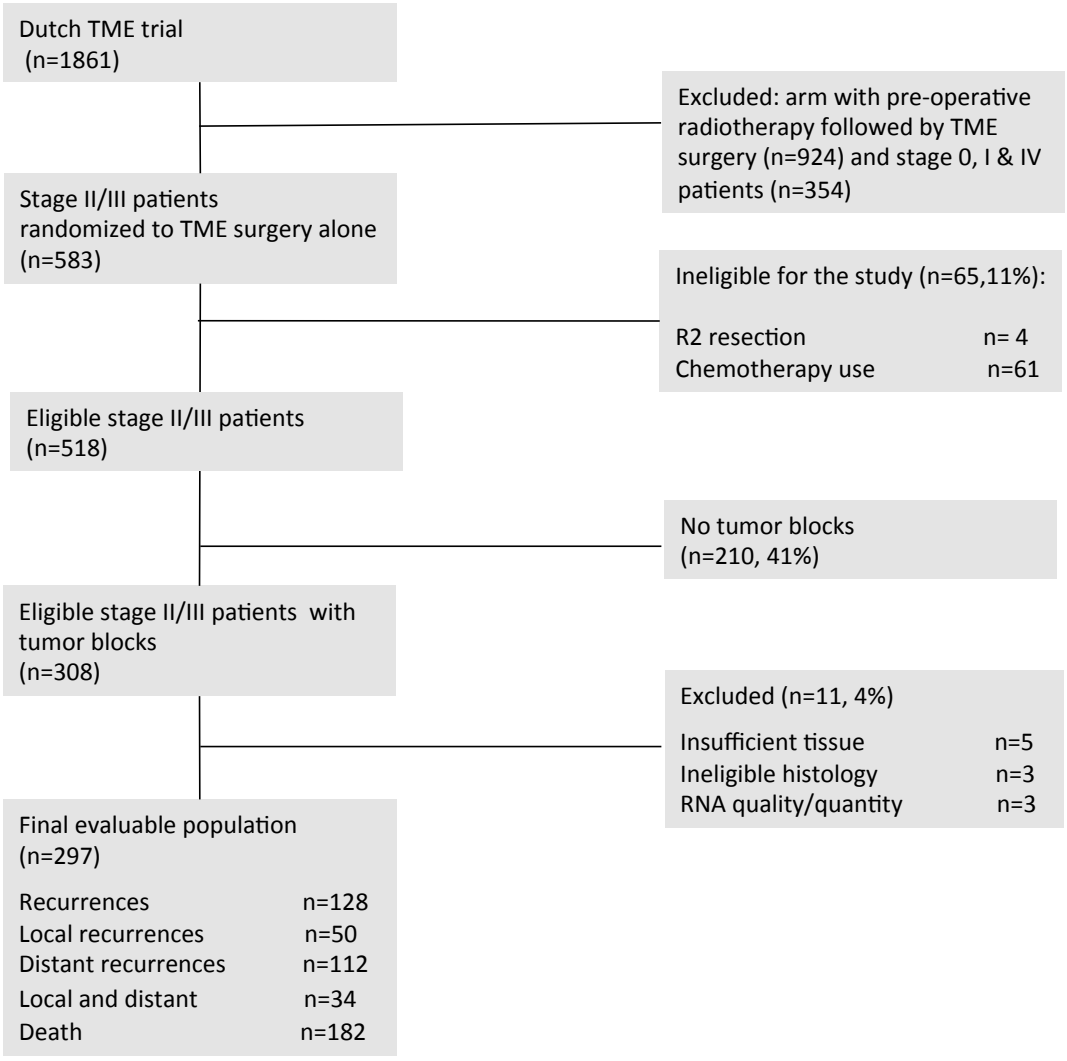


Figure 1: Study flow diagram.
TME, Total Mesorectal Excision

stage IIIC patients. A total of 182 patients died, including 120 (66%) patients who died after recurrence of rectal cancer.

Patient characteristics were representative of a contemporary rectal cancer population, with median age of 66 (range 23-92), the majority being male (63%), and receiving a low anterior resection (LAR) (64%) (Table I). Most patients had T3-T4 tumors (90%) and 30% of the tumors were high grade. The median number of nodes examined was 9 (range 1-52) and 36% of the patients had ≥12 nodes examined (Table I). Importantly, a quarter of patients had positive resection margins, with the proportion of RM-positive patients increasing from 16% in stage II to 53% in stage IIIC (Table I).

The demographic and pathologic characteristics of patients evaluated in this study were similar to those of eligible stage II and III patients in the parent trial without FPE tissue (Supplemental Table I). RFI was comparable as well (logrank *p*-value 0.507).

Table I: Baseline Clinical and Pathologic Characteristics for the total cohort and stratified for stage.

Characteristic	Values	All N(%) 297 pts	Stage II (N%) 130 pts	Stage III A/B (N%) 110 pts	Stage III C (N%) 57 pts
Year of surgery	<1998	157 (52.9)	69 (53.1)	56 (50.9)	32 (56.1)
	≥1998	140 (47.10	61 (46.9)	54 (49.1)	25 (43.9)
Age	<60	102 (34.3)	50 (38.5)	35 (31.8)	17 (29.8)
	60 to <70	89 (30.0)	33 (25.4)	41 (37.3)	15 (26.30
	70+	106 (35.7)	47 (36.2)	34 (30.9)	25 (43.9)
Gender	Female	111 (37.4)	56 (43.1)	34 (30.9)	21 (36.8)
	Male	186 (62.6)	74 (56.9)	76 (69.1)	36 (63.2)
Resection type	LAR	191 (64.3)	80 (61.5)	77 (70.0)	34 (59.6)
	APR	106 (35.7)	50 (38.5)	33 (30.0)	23 (40.4)
Resection margin status	R0	223 (75.1)	109 (83.8)	87 (79.1)	27 (47.4)
	R1 no RT	37 (12.5)	15 (11.5)	9 (8.2)	13 (22.8)
	R1+RT	37 (12.5)	6 (4.6)	14 (12.7)	17 (29.8)
Distance from anal verge*	<5 cm	103 (34.7)	49 (37.7)	36 (32.7)	18 (31.6)
	5-9.9 cm	110 (37.0)	42 (32.3)	49 (44.5)	19 (33.3)
	10+ cm	84 (28.3)	39 (30.0)	25 (22.7)	20 (35.1)
T-Stage	T1	1 (0.3)		1 (0.9)	0 (0.0)
	T2	29 (9.8)		22 (20.0)	7 (12.3)
	T3	248 (83.5)	123 (94.6)	82 (74.5)	43 (75.4)
	T4	19 (6.4)	7 (5.4)	5 (4.5)	7 (12.3)
Number of lymph nodes examined	<12	190 (64.0)	95 (73.1)	74 (67.3)	21 (36.8)
	12+	107 (36.0)	35 (26.9)	36 (32.7)	36 (63.2)
Grade **	High	88 (29.6)	22 (16.9)	38 (34.5)	28 (49.1)
	Low	209 (70.4)	108 (83.1)	72 (65.5)	29 (50.9)
Tumour type	Mucinous	16 (5.4)	4 (3.1)	8 (7.3)	4 (7.0)
	Adenocarcinoma	281 (94.6)	126 (96.9)	102 (92.7)	53 (93.0)
Obstruction or perforation	Present	21 (7.1)	7 (5.4)	6 (5.5)	8 (14.0)
	Absent	276 (92.9)	123 (94.6)	104 (94.5)	49 (86.0)

Abbreviations: RT=Radiotherapy, R0= Radical resection, R1: residual disease after resection

** To inferior margin of tumor*

*** Centrally assessed by a pathologist at Genomic Health*

Association of Recurrence Score Result with Outcomes

Recurrence Score values ranged from 0 to 72 with a median score of 32 (interquartile range, 24 to 42) and a mean ± SD of 33.3 ± 12.7. In the primary analysis, the continuous Recurrence Score result was significantly associated with recurrence risk, when controlling for stage and RM status, with a hazard ratio (HR) of 1.57 for a 25-unit increase in the score (95% CI 1.11-2.21, p=0.011). The proportional hazards assumption held (p=0.52). An interaction between Recurrence Score result and stage was observed (p=0.002), with evidence of nonlinearity in the relationship between the continuous score and the log hazard of recurrence (p<0.001). Adjusting for stage and RM status and accounting for interaction with stage and non-linearity, the Recurrence Score result was associated

with risk of recurrence in stage II (HR defined as ratio of the hazards at the 75th and 25th percentile of RS, 3.27, 95% CI 1.52-7.01, $p<0.001$) and stage IIIA/B (HR, 1.87, 95% CI 1.18-2.95, $p=0.007$) (Figure 2). The Recurrence Score result was not associated with recurrence risk in stage IIIC (HR, 0.75, 95% CI 0.46-1.21, $p=0.243$). The pre-defined high Recurrence Score group had higher recurrence risk than the low group in stage II (HR, 5.81, 95% CI 2.33-14.50, $p<0.001$) but not in stage IIIA/B (HR, 1.62, 95% CI 0.82-3.19, $p=0.169$) or stage IIIC (HR, 0.64, 95% CI 0.29-1.41, $p=0.272$): the effect of the Recurrence Score was most prominent in stage II and attenuated in more advanced stage (Figure 3). In the stage II patients, cumulative incidence estimates of 5-year recurrence risk for the low- (63 patients, 48%), intermediate- (37 patients, 28%), and high (30 patients, 23%) Recurrence Score groups were 11% (95% CI 6-22%), 27% (95% CI 16-46%) and 43% (95% CI 29-65%), respectively (Table II). Recurrence risk estimates by Kaplan-Meier methods were similar for low group and higher for the high score group (Supplemental Table II and Figure 1).

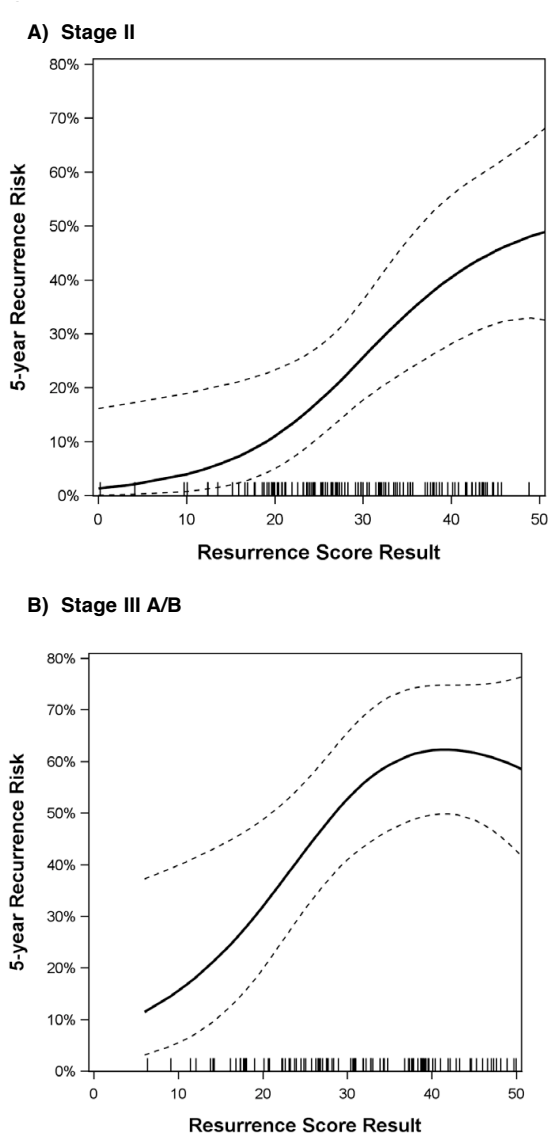
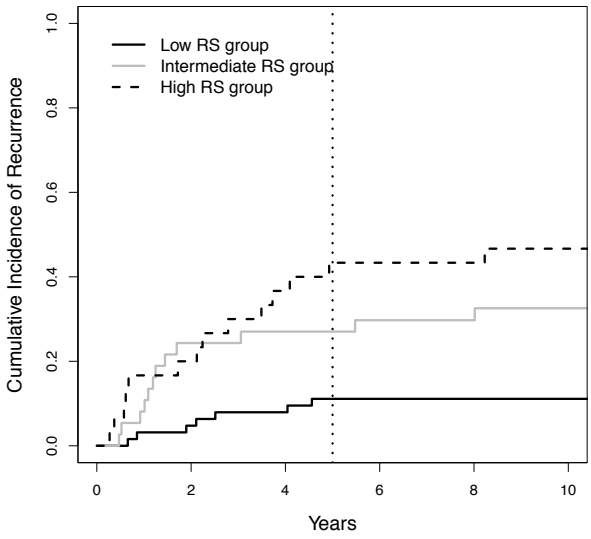
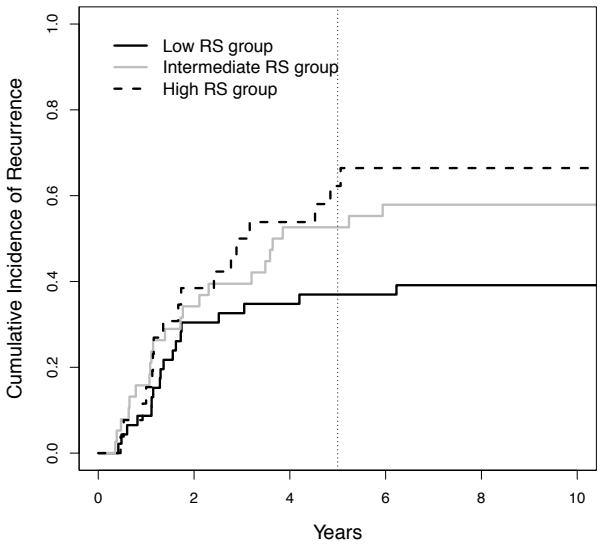


Figure 2: Relationship between risk of recurrence and continuous Recurrence Score in patients with negative resection margins.
Relationship between risk of recurrence and continuous Recurrence Score in 297 rectal cancer patients with negative resection margins. A) stage II, B) stage IIIA/B (1-3 positive lymph nodes). The solid line represents risk of recurrence; the dotted lines represent 95% confidence intervals. A rug plot depicting the distribution of Recurrence Score values is included at the bottom of each figure.

A



B



C

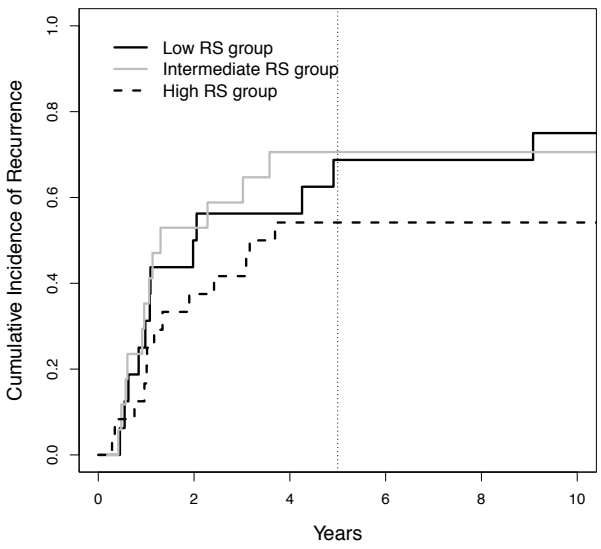


Figure 3: Cumulative incidence for recurrence by stage and Recurrence Score group Cumulative incidence curves for recurrence in 297 rectal cancer patients by Recurrence Score group based on prespecified cut-points and separated by stage. A) stage II, B) stage IIIA/B (1-3 positive lymph nodes), C) stage IIIC (4 or more positive lymph nodes).
Solid black line represents low Recurrence Score group, solid grey line – intermediate Recurrence Score group and dashed black line - high Recurrence Score group.

Table II: Five-year Estimates of Cumulative Incidence in Stage II Rectal Cancer Patients (n=130)

Recurrence Score group	N (%) pts	Cumulative Incidence for Recurrence (95% CI)	Cumulative Incidence for Distant Recurrence (95% CI)	Cumulative Incidence for Rectal Cancer Specific Mortality (95% CI)
Low	63 (48.5%)	11.1% (5.5%, 22.3%)	7.9% (3.4%, 18.4%)	4.8% (1.6%, 14.4%)
Intermediate	37 (28.5%)	27.0% (15.9%, 45.8%)	24.3% (13.8%, 42.9%)	18.9% (9.7%, 36.9%)
High	30 (23.1%)	43.3% (28.8%, 65.2%)	33.3% (20.1%, 55.2%)	30.0% (17.4%, 51.8%)

Similar results were observed for DRFI and RCSS: in the pre-specified main-effects models, the Recurrence Score result was significantly associated with DRFI (HR for 25 unit increase in the score of 1.50, 95% CI 1.04-2.17, p=0.030) and RCSS (HR of 1.64 (95% CI 1.15-2.34, p=0.007). Significant interaction between Recurrence Score result and stage and non-linearity were also observed for these endpoints. In stage II patients, cumulative incidence estimates of 5-year recurrence ranged from 8% (95% CI 3-18%) to 33% (95% CI 20-55%) for DRFI and from 5% (95% CI 2-14%) to 30% (95% CI 17-52%) for RCSS for low vs. high score groups, respectively (Table II).

The Recurrence Score result was not significantly associated with DFS (p=0.118) and OS (p=0.111) in the pre-specified analyses, similar to one of the colon cancer validation studies [13] where most deaths were not cancer-related. Notably, in this study, 52% of deaths in stage II patients were not due to rectal cancer.

Recurrence Score in the Context of Conventional Clinical and Pathologic Factors

When clinical and pathologic factors were examined (Supplemental Table III), higher age (p=0.041) and higher T-stage (T4N0, T3-4N1 vs. T3N0, T1-2N1, p=0.016) were associated with recurrence in analyses adjusted for stage and resection margin. Type of surgical resection and distance from anal verge showed an interaction with stage (p=0.026 and p=0.049, respectively), with LAR and greater distance from the anal verge associated with lower risk of recurrence in stage IIIC (both p<0.005) but not in stage II or stage IIIA/B. While resection margin status was significantly associated with outcome in the univariate analysis (p=0.015), its effect was attenuated after adjustment for stage in the multivariable analyses, paralleling what was observed for resection margin status in all eligible stage II-III surgery alone patients in the TME trial.

In pre-specified multivariable analysis adjusted for stage, RM status, T-stage, grade and number of nodes examined, the Recurrence Score result was a significant predictor of recurrence risk in stage II (p<0.001) and stage IIIA/B (p=0.019), but not Stage IIIC (p=0.122) (Table III). Similar results were observed when age, the only other covariate associated with RFI, was added to the model, and when the analysis was adjusted for circumferential (radial) margin status only. The model with Recurrence Score and

Table III: Multivariable Analysis: Contribution of Recurrence Score to Prediction of Recurrence Risk beyond Clinical and Pathologic Covariates

Variable	HR	HR (95% CI)	*p*-value
Stage			
IIIA/B vs. II	1.36	(0.71-2.95)	0.36
IIIC vs. II	2.48	(1.22-5.02)	0.01
Resection margin status			
R1 no RT vs. R0	1.02	(0.59-1.75)	0.95
R1 + RT vs.R0	1.01	(0.62-1.67)	0.96
T-Stage			
T4N0, T3-4N1 vs. T3N0, T1-2N1	2.03	(1.14-3.60)	0.01
Grade *			
High vs. low	0.99	(0.67-1.45)	0.95
Number of nodes examined			
12+ vs. <12	1.10	(0.75-1.62)	0.63
RS contribution**			
RS in stage II	3.40	(1.58-7.30)	<0.001
RS in stage IIIA/B	1.75	(1.11-2.77)	0.02
RS in stage IIIC	0.69	(0.42-1.12)	0.12

** Centrally assessed by a pathologist at Genomic Health*

*** Includes stage specific linear and spline terms (2 d.f.) to account for non-linearity. Hazard Ratio for Recurrence Score is the ratio of the hazards at the 75th and 25th percentiles of Recurrence Score*

conventional measures identified 25% of stage II patients with 5-year recurrence risk below 15% and 39% of patients with risks above 30% while the model based on the conventional measures alone assessed the risk for 95% of stage II patients to be in the 15%-30% range and 5% of patients with risk above 30%. Addition of the Recurrence Score assay to conventional measures resulted in higher relative utility (Figure 4). A test tradeoff calculation [21] illustrates the value of the assay for different treatment paradigms. If default strategy is treating everyone, testing 14 to 18 patients is required for every correct prediction of recurrence to increase the net benefit of risk prediction compared to conventional measures alone (risk thresholds 25-30%). If therapy is not routinely recommended, testing 37 to 45 patients is required (risk thresholds 45-50%).

The Recurrence Score result predicted DRFI (stage II p=0.009, stage IIIA/B p=0.020) and RCSS (stage II p<0.001 and stage IIIA/B p=0.034) after adjustment for these additional covariates.

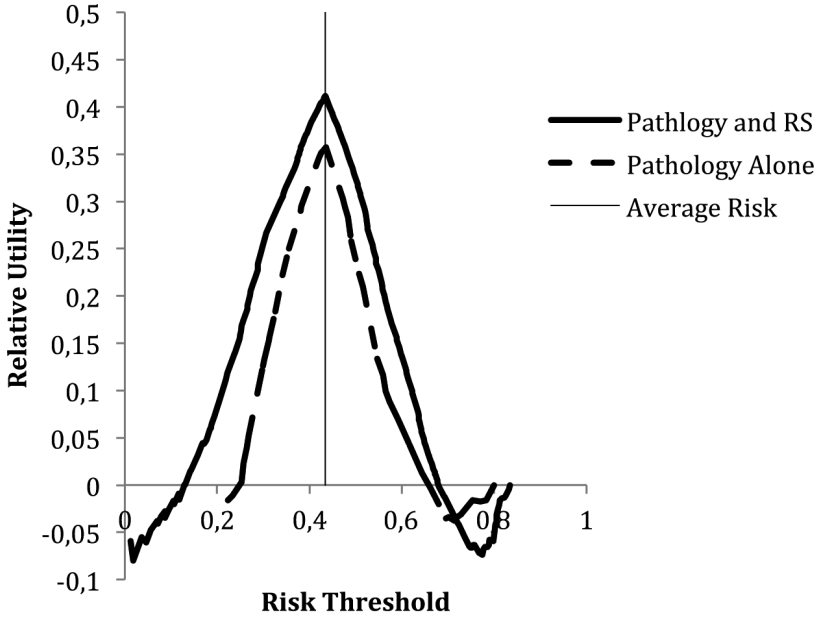


Figure 4: Relative utility curves for recurrence risk prediction using the models with and without Recurrence Score in all patients.

Relative utility curves for recurrence risk prediction in 297 rectal cancer patients. Relative utility is the maximum net benefit of prediction divided by the net benefit of perfect prediction. Risk threshold is the recurrence risk at which a patient is indifferent to the use of a treatment (e.g. post-operative chemotherapy). Solid black line represents Cox regression model with Recurrence score, N and T stage, resection margin status, number of nodes examined and grade. Dashed black line represents Cox regression model with N and T stage, resection margin status, number of nodes examined and grade.

DISCUSSION

In this prospectively-designed study, the 12-gene Recurrence Score was validated as a predictor of recurrence in stage II and III rectal cancer patients treated with TME surgery alone, providing information beyond conventional clinical and pathologic factors [12-14]. There was a significant interaction between Recurrence Score and stage, with the Recurrence Score providing the greatest discrimination of recurrence risk in stage II disease and little discrimination in stage IIIC.

Consistency of these rectal cancer results with 3 large validation studies of the Recurrence Score assay in colon cancer supports the association of this score with metastatic potential of large bowel cancers, and demonstrates the presence of common biological determinants of recurrence across tumors arising from the colon as well as the rectum.

Improved risk discrimination with the Recurrence Score result in stage II and IIIA/B rectal cancer should have clinical relevance for patients and physicians considering individualized approaches to pre-operative and post-operative treatment. In the United States the standard recommendation for treatment of stage II and III rectal

cancer patients includes neoadjuvant chemoradiation followed by TME surgery and postoperative adjuvant chemotherapy, based on extrapolation from trials in colon cancer [22;23]. By contrast, in most countries in Europe, adjuvant chemotherapy is not routinely recommended in rectal cancer. The benefit of adjuvant chemotherapy in patients with combined chemoradiation before surgery is controversial [7-10]. Across these treatment paradigms, the ability of Recurrence Score to identify patients with widely different risks of recurrence may enable tailored approaches, directing use of pre-operative and post-operative chemotherapy and radiation to patients at high risk of tumor recurrence and less aggressive treatment for low risk patients. In this regard, the low recurrence risk observed in our study for the large sub-group of stage II rectal cancer patients with low Recurrence Score results may be particularly impactful, as these patients demonstrated excellent outcomes without any pre- or post-operative chemotherapy or radiation. In moderate risk patients, the decision for more aggressive treatment should be discussed by patient and physician taking into account potential recurrence risk, morbidity associated with treatment, comorbidities and patient preferences. It is important to note that the ability of the Recurrence Score to predict neoadjuvant or adjuvant chemotherapy benefit in rectal cancer has not been studied. This study focused on patients who did not receive pre-operative chemotherapy or radiation, and the assay's ability to differentiate risk for patients with neoadjuvant therapies should be addressed in future studies.

The results of this validation study are consistent with recent analyses by the Cancer Genome Atlas Network [24], demonstrating similarity of colon and rectal cancers at the genomic level. A number of recent studies have suggested the existence of different subtypes of colorectal cancer [25-29]. All support the notion that colorectal tumors with a stromal response signature (EMT/TGFbeta signalling) have the worst outcome. Our results reaffirm the clinical relevance of two key biological pathways measured by the Recurrence Score assay - stromal response and cell cycle control, which is consistently reflected across multiple subtyping and genomic profiling efforts in the literature.

This prospectively-designed validation study demonstrates that the 12-gene colon cancer assay, can assess risk of recurrence in rectal cancer patients. The low exclusion rate observed during sample processing was consistent with QUASAR (3.6%), CALGB (3.1%) and C-07 (3.1%), indicating a precise and robust analytical process [12-14]. Limitations should also be acknowledged. First, blocks for only 59% of eligible patients were collected, although the demographics for those with blocks and without blocks were similar. Second, risk discrimination by Recurrence Score was attenuated in stage IIIA/B and IIIC, and Recurrence Score was not an independent recurrence risk predictor in stage IIIC. The reason for this attenuation is unclear, but may relate to challenges with achieving a complete resection of tumor at higher stage, which may affect recurrence rates beyond the biology of the tumor itself. Furthermore, the total study size is modest in absolute numbers and some subgroup analyses may be underpowered, but this is

one of the largest cohorts of well-characterized rectal cancer patients to be studied with a gene expression assay.

The use of adjuvant chemotherapy in rectal cancer is still under debate, and efforts are underway to study reduced-intensity approaches, including those that spare radiation and even surgery. Incorporation of the Recurrence Score assay into clinical trials, along the lines of the TAILORx and RxPonder trials in breast cancer[30;31], may enable these efforts through improved patient stratification for risk-adapted treatment strategies. Our results highlight the importance of understanding the underlying biology of rectal tumors for individual patients in assessing risk and potentially guiding treatment decisions in this disease.

REFERENCE LIST

(1) Kapiteijn E, Marijnen CA, Colenbrander AC et al. Local recurrence in patients with rectal cancer diagnosed between 1988 and 1992: a population-based study in the west Netherlands. *Eur J Surg Oncol* 1998;24:528-535.

(2) Kapiteijn E, Kranenbarg EK, Steup WH et al. Total mesorectal excision (TME) with or without preoperative radiotherapy in the treatment of primary rectal cancer. Prospective randomised trial with standard operative and histopathological techniques. Dutch ColoRectal Cancer Group. *Eur J Surg* 1999;165:410-420.

(3) Kapiteijn E, Marijnen CA, Nagtegaal ID et al. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. *N Engl J Med* 2001;345:638-646.

(4) Peeters KC, Marijnen CA, Nagtegaal ID et al. The TME trial after a median follow-up of 6 years: increased local control but no survival benefit in irradiated patients with resectable rectal carcinoma. *Ann Surg* 2007;246:693-701.

(5) van GW, Marijnen CA, Nagtegaal ID et al. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer: 12-year follow-up of the multicentre, randomised controlled TME trial. *Lancet Oncol* 2011;12:575-582.

(6) Petersen SH, Harling H, Kirkeby LT, Wille-Jorgensen P, Mocellin S. Postoperative adjuvant chemotherapy in rectal cancer operated for cure. *Cochrane Database Syst Rev* 2012;3:CD004078.

(7) Bosset JF, Collette L, Calais G et al. Chemotherapy with preoperative radiotherapy in rectal cancer. *N Engl J Med* 2006;355:1114-1123.

(8) Bujko K, Glynne-Jones R, Bujko M. Adjuvant chemotherapy for rectal cancer. *Ann Oncol* 2010;21: 2443.

(9) Glimelius B, Dahl O, Cedermark B et al. Adjuvant chemotherapy in colorectal cancer: a joint analysis of randomised trials by the Nordic Gastrointestinal Tumour Adjuvant Therapy Group. *Acta Oncol* 2005;44:904-912.

(10) Gray R, Barnwell J, McConkey C, Hills RK, Williams NS, Kerr DJ. Adjuvant chemotherapy versus observation in patients with colorectal cancer: a randomised study. *Lancet* 2007;370:2020-2029.

(11) O'Connell MJ, Lavery I, Yothers G et al. Relationship between tumor gene expression and recurrence in four independent studies of patients with stage II/III colon cancer treated with surgery alone or surgery plus adjuvant fluorouracil plus leucovorin. *J Clin Oncol* 2010;28:3937-3944.

(12) Gray RG, Quirke P, Handley K et al. Validation study of a quantitative multigene reverse transcriptase-polymerase chain reaction assay for assessment of recurrence risk in patients with stage II colon cancer. *J Clin Oncol* 2011;29:4611-4619.

(13) Venook AP, Niedzwiecki D, Lopatin M et al. Biologic determinants of tumor recurrence in stage II colon cancer: validation study of the 12-gene recurrence score in cancer and leukemia group B (CALGB) 9581. *J Clin Oncol* 2013;31:1775-1781.

(14) Yothers G, O'Connell MJ, Lee M et al. Validation of the 12-Gene Colon Cancer Recurrence Score in NSABP C-07 As a Predictor of Recurrence in Patients With Stage II and III Colon Cancer Treated With Fluorouracil and Leucovorin (FU/LV) and FU/LV Plus Oxaliplatin. *J Clin Oncol* 2013.

(15) Yerushalmi R, Woods R, Ravdin PM, Hayes MM, Gelmon KA. Ki67 in breast cancer: prognostic and predictive potential. *Lancet Oncol* 2010;11:174-183.

(16) Compton CC, Fielding LP, Burgart LJ et al. Prognostic factors in colorectal cancer. College of American Pathologists Consensus Statement 1999. *Arch Pathol Lab Med* 2000;124:979-994.

(17) Clark-Langone KM, Sangli C, Krishnakumar J, Watson D. Translating tumor biology into personalized treatment planning: analytical performance characteristics of the Oncotype DX Colon Cancer Assay. *BMC Cancer* 2010;10:691.

(18) Stone C.J., Koo C.Y. Additive splines in statistics. Proc Stat Comp Sect Am Statist Assoc , 45-48. 1985.

(19) Putter H, Fiocco M, Geskus RB. Tutorial in biostatistics: competing risks and multi-state models. *Stat Med* 2007;26:2389-2430.

(20) Baker SG. Putting risk prediction in perspective: relative utility curves. *J Natl Cancer Inst* 2009;101: 1538-1542.

(21) Baker SG, Schuit E, Steyerberg EW et al. How to interpret a small increase in AUC with an additional risk prediction marker: decision analysis comes through. *Stat Med* 2014.

(22) Glimelius B, Oliveira J. Rectal cancer: ESMO clinical recommendations for diagnosis, treatment and follow-up. *Ann Oncol* 2009;20 Suppl 4:54-56.

(23) Kuebler JP, Wieand HS, O'Connell MJ et al. Oxaliplatin combined with weekly bolus fluorouracil and leucovorin as surgical adjuvant chemotherapy for stage II and III colon cancer: results from NSABP C-07. *J Clin Oncol* 2007;25:2198-2204.

(24) Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 2012;487: 330-337.

(25) Budinska E, Popovici V, Tejpar S et al. Gene expression patterns unveil a new level of molecular heterogeneity in colorectal cancer. *J Pathol* 2013;231:63-76.

(26) De Sousa E Melo, Wang X, Jansen M et al. Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. *Nat Med* 2013;19:614-618.

(27) Loboda A, Nebozhyn MV, Watters JW et al. EMT is the dominant program in human colon cancer. *BMC Med Genomics* 2011;4:9.

(28) Marisa L, de RA, Duval A et al. Gene expression classification of colon cancer into molecular subtypes: characterization, validation, and prognostic value. *PLoS Med* 2013;10:e1001453.

(29) Perez-Villamil B, Romera-Lopez A, Hernandez-Prieto S et al. Colon cancer molecular subtypes identified by expression profiling and associated to stroma, mucinous type and different clinical behavior. *BMC Cancer* 2012;12:260.

(30) Ramsey SD, Barlow WE, Gonzalez-Angulo AM et al. Integrating comparative effectiveness design elements and endpoints into a phase III, randomized clinical trial (SWOG S1007) evaluating oncotypeDX-guided management for women with breast cancer involving lymph nodes. *Contemp Clin Trials* 2013;34:1-9.

(31) Sparano JA, Paik S. Development of the 21-gene assay and its application in clinical practice and clinical trials. *J Clin Oncol* 2008;26:721-728.

SUPPLEMENTAL MATERIAL

Supplemental Table I: Comparison of patient characteristics for eligible patients with and without blocks from the TME trial

Characteristic	Values	Evaluable patients in this study (N=297)	Eligible TME trial patients without blocks (N=210)	*p*-value*
Age	<60	102 (34.3)	58 (27.6)	0.07
	60 to <70	89 (30.0)	63 (30.0)	
	70+	106 (35.7)	89 (42.4)	
Gender	Female	111 (37.4)	83 (39.5)	0.62
Number of Nodes Examined	<12	190 (64.0)	133 (63.9)	0.99
Number of	0 (Stage II)	130 (43.8)	111 (53.4)	0.17
Nodes Involved	1-3 (Stage IIIA/B)	110 (37.0)	57 (27.4)	
	4+ (Stage IIIC)	57 (19.2)	40 (19.2)	
T-Stage	T1-T2	30 (10.1)	14 (6.7)	0.85
	T3	248 (83.5)	190 (90.9)	
	T4	19 (6.4)	5 (2.4)	
Obstruction or Perforation	Present	21 (7.1)	9 (4.3)	0.19
Grade**	High	73 (24.6)	35 (28.2)	0.43
Resection margin	R1	74 (24.9)	38 (18.3)	0.08

** p-values are from the chi-square and Cochran-Mantel-Haenszel chi-square tests for nominal categorical and ordered categorical variables, respectively*

*** Locally assessed during TME trial; available for 124 patients without blocks.*

Supplemental Table II: Five-year Estimates of Risk based on Kaplan Meier analysis in Stage II Rectal Cancer Patients (n=130)

Recurrence Score group	N (%) pts	Recurrence Risk (95% CI)	Distant Recurrence Risk (95% CI)	Rectal Cancer Specific Mortality (95% CI)
Low	63 (48.5%)	12.4% (6.1%, 24.3%)	9.1% (3.9%, 20.4%)	5.3% (1.8%, 15.7%)
Intermediate	37 (28.5%)	28.7% (16.6%, 46.8%)	25.8% (14.4%, 43.8%)	20.3% (10.2%, 37.9%)
High	30 (23.1%)	52.7% (34.7%, 73.2%)	45.9% (27.6%, 68.8%)	37.0% (21.2%, 59.1%)

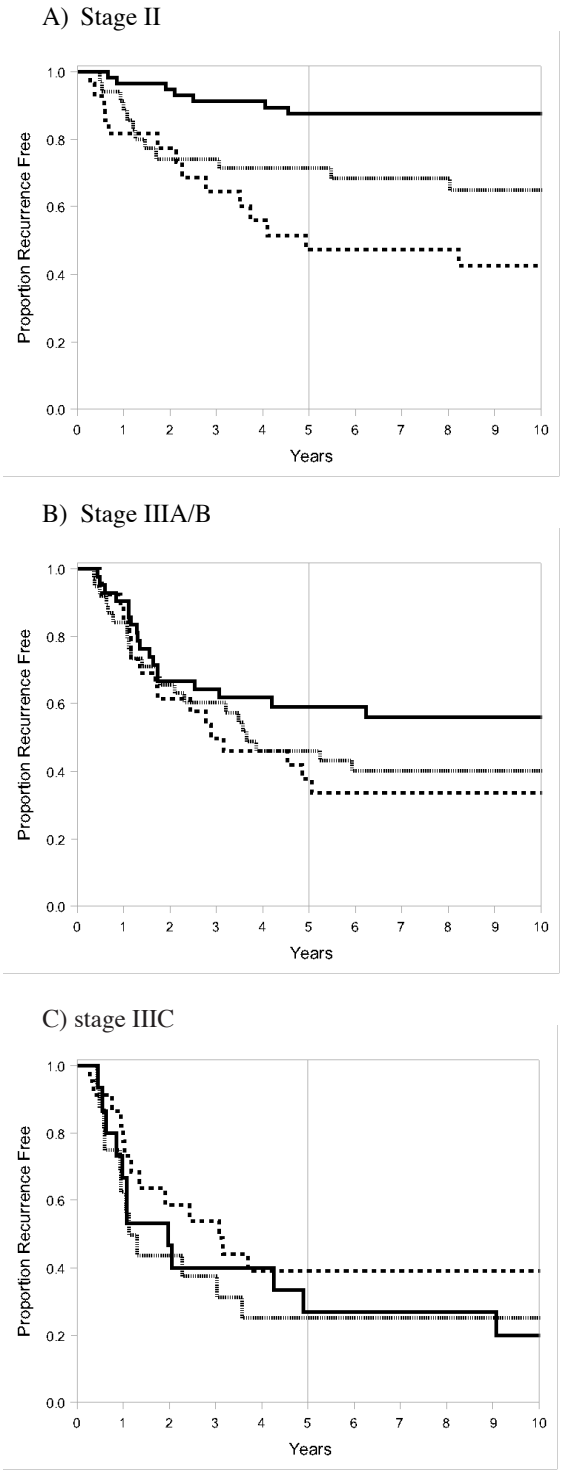
Supplemental Table III: Association of conventional clinical and pathologic factors with risk of recurrence.

Characteristic	Values	HR	HR 95% CI	*p*-value*	*p*-value for interaction with stage***
Age	Continuous, per 1 year increase	1.02	(1.00,1.03)	0.04	0.92
Grade, central	High vs low	1.01	(0.68,1.49)	0.96	0.61
Grade, local	High vs low	1.06	(0.71,1.57)	0.78	0.74
Nodes examined	<12 vs. 12+	1.18	(0.80,1.74)	0.40	0.67
Gender	Male vs. Female	1.09	(0.75,1.58)	0.64	0.58
T Stage	T4N0, T3-4N1 vs. T3N0, T1-2N1	1.89	(1.10,3.25)	0.02	0.28
Surgery	APR vs. LAR	1.44	(1.00,2.06)	0.05	0.03
Distance from anal verge	5-9.9 vs. <5	0.93	(0.62,1.39)	0.72	0.05
	10+ vs. <5	0.62	(0.39,0.99)	0.04	
Residual disease**	R1 vs. R0	1.28	(0.86,1.92)	0.23	0.30
Resection margin status**	R1 no RT vs. R0	1.18	(0.69,2.04)	0.55	0.52
	R1 + RT vs. R0	1.37	(0.85,2.22)	0.21	
CRM margin (<1 mm)**	Positive vs Negative	1.34	(0.89,2.00)	0.17	0.27
CRM margin (<2 mm)**	Positive vs Negative	1.28	(0.87,1.87)	0.21	0.21

**Based on Cox PH models including a given covariate, stage and RM status.*

***Based on Cox PH models including a given covariate and stage.*

****Based on Cox PH models including a given covariate, stage and interaction of covariate and stage.*



Supplemental Figure 1: Kaplan Meier analysis for recurrence-free interval by stage and Recurrence Score group.

Kaplan Meier curves for Recurrence Free Interval (RFI) in 297 rectal cancer patients stratified for Recurrence Score group based on prespecified cut-points and separated by stage. A) stage II, B) stage IIIA/B (1-3 positive lymph nodes), C) stage IIIC (4 or more positive lymph nodes).

Solid black line represents low Recurrence Score group, dashed black/grey line-intermediate Recurrence Score group and dotted black line-high Recurrence Score group.

PART TWO

Treatment of colon cancer and predictive biomarkers

CHAPTER 6

Aspirin use after diagnosis improves survival in older adults with colon cancer: a retrospective cohort study

Marlies S. Reimers, Esther Bastiaannet, Myrthe P.P. van Herk-Sukel, Valery E.P. Lemmens, Colette B.M. van den Broek, Cornelis J.H. van de Velde, Anton J.M. de Craen, Gerrit Jan Liefers

Journal of the American Geriatrics Society, 2012 Dec;60(12):2232-6

ABSTRACT

Background

Preclinical studies have shown aspirin might prolong survival due to inhibition of tumor growth and metastases in colon cancer patients. To date, however, it is unclear whether aspirin, prescribed as an adjuvant therapy, can influence the prognosis of colon cancer patients. An effective and well-tolerated adjuvant therapy would be a major clinical advancement, particularly in older cancer patients. The aim of this study was to assess survival in relation to aspirin use after diagnosis in older colon cancer patients.

Methods

Subgroup analysis of a previously published cohort and retrospective study of 536 patients aged 70 years and older diagnosed with colon cancer registered in the Eindhoven Cancer Registry (ECR) between 1998 and 2007, linked to prescriptions of low dose aspirin (80 mg) registered in the community pharmacy database of the PHARMO record linkage system.

Survival was analyzed with user status as a time-dependent covariate. Multivariable Poisson regression survival models were used to study the effect of aspirin on Overall Survival (OS).

Results

Overall, 107 patients (20.0%) started aspirin after being diagnosed with colon cancer; 429 patients (80.0%) were not prescribed aspirin. In total 339 patients (63.2%) died at the end of follow up. Aspirin use after diagnosis was associated with a better OS with a Rate Ratio (RR) of 0.51 (95% CI 0.38-0.70 $p<0.001$). Multivariable proportional hazards regression analysis revealed aspirin use was associated with overall survival (adjusted RR 0.59 (95% CI 0.44-0.81, $p=0.001$)).

Conclusions

Aspirin use after the diagnosis of colon cancer in older patients was associated with better survival. These results suggest that low dose aspirin could be used as an effective adjuvant therapy in older colon cancer patients.

INTRODUCTION

Nearly half of all patients with colon cancer are above 70 years of age and this age group is expanding as a result of increasing life expectancy [2]. Approximately fifty percent of all patients undergoing colorectal cancer surgery are known to develop a relapse and die of metastatic disease [1;3;4]. The introduction of adjuvant chemotherapy has significantly improved the prognosis of colon cancer patients. However, the effect of adjuvant chemotherapy on older patients is less clear. Some studies have suggested lack of survival benefit with adjuvant chemotherapy in patients older than 65 years [5]. Other studies, however, have suggested that older patients do benefit similar from chemotherapy, but that they are less frequently treated [4;6]. Undertreatment with adjuvant chemotherapy of older patients often occurs because of co-morbidities and patient preferences [7]. Due to underrepresentation in clinical trials there is no treatment consensus for elderly patients with colon cancer.

Aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) are effective in preventing colorectal cancer [8-10]. Aspirin inhibits cyclooxygenase-2 (COX-2), which is expressed in 70% of the colorectal tumors and increases with disease stage [11;12]. COX-2 plays an important role in colorectal carcinogenesis, invasion, angiogenesis and metastasis. Several studies have shown that this COX-2 effect can be reversed by selective COX-2 inhibitors [13]. It is not clear whether aspirin can influence the prognosis of patients with colorectal cancer, but in animal models aspirin and NSAIDs with activity against the COX-2 isoenzyme have shown to inhibit tumor progression and increase survival [14]. Besides, clinical studies have shown an association between aspirin and prognosis as well. A recent study in patients with stage I-III colorectal cancer selected from two nationwide health professional cohorts in the U.S. showed that regular aspirin use after the diagnosis of colorectal cancer compared with non-users was associated with a lower risk of colorectal cancer-specific and overall mortality, especially among individuals with tumors that overexpress COX-2 [11].

The number of colon cancer patients is increasing and there is a strong need for therapeutic improvement, especially in elderly patients, who are less frequently treated with standard chemotherapy. The aim of this study was to assess the association of aspirin use after the diagnosis of colon cancer on survival in patients aged 70 years and older.

METHODS

Patients

The central patient database of PHARMO, which links to more than 10 databases using different medical record linkage algorithms, was recently combined with data from the

Eindhoven Cancer Registry (ECR) [15]. From the PHARMO database, prescriptions of low dose aspirin (80 mg) were selected and linked to patients diagnosed with colorectal cancer, registered in the ECR between 1998 and 2007. In total, 4481 colorectal cancer patients were included in this database. We performed a subgroup analysis on this previously published cohort, comprising specifically patients 70 years and older, diagnosed with colon cancer, who used aspirin only after diagnosis or who never used aspirin (n=536).[1] The date of prescription and date of diagnosis were compared to assess whether the aspirin was prescribed only after the diagnosis. Nonusers were defined as patients who were never used prescribed aspirin. Patients who were prescribed aspirin after diagnosis were defined as users.

Statistics

Vital status of patients was established either directly from the patient's medical record or through linkage of cancer registry data with the municipal population registries, which record information on the vital status of their inhabitants. Follow-up started at 30 days from diagnosis of colorectal cancer (T0), as information concerning the prescriptions in hospital was unknown. Follow-up was until the last contact date or date of death. Users were defined as patients who had at least 1 prescription for aspirin for at least 14 days; patients who were prescribed aspirin for less than 14 days were defined as nonusers. Time-dependent survival analyses were used to assess survival. Patients were defined as nonusers from T0 to first use and user from first use to the end of the follow-up. Poisson regression survival models were used to study the effect of aspirin on overall survival. In multivariable proportional hazards regression analysis adjustments were made for sex, age (continuous), stage (pathological stage and clinical stage if pathological stage was unknown), adjuvant chemotherapy (yes/no), co-morbidity (yes/no), surgery (yes/no), grade, localization of the tumor, and year of diagnosis. Finally, stratified analyses were performed for type of co-morbidity, chemotherapy, grade, stage, surgery and localization of the tumor.

RESULTS

Overall, 536 patients aged 70 years and older diagnosed with colon cancer between 1998 and 2007 were included in the analyses. There were 107 patients (20%) who started low-dose aspirin (80 mg) after diagnosis and 429 patients (80%) who did not use prescribed aspirin before or after diagnosis. Table I shows the patient baseline characteristics; Median age was 77.6 (SD 5.3) years. Patients who used aspirin were significantly younger than patients without aspirin use. Non-users were more likely to be diagnosed with stage IV colon cancers compared to aspirin users. Also, aspirin users were more often diagnosed

Table I: Baseline Characteristics of Study Population

	Overall N=536	%	Aspirin + N=107	%	Aspirin – N=429	%	*P*-value
Age,Mean (SD[a])	77.6 (5.3)		76.6 (4.9)		77.8 (5.4)		0.04
Sex							0.57
Female	258	48.1	48	44.9	210	49.0	
Male	278	51.9	59	55.1	219	51.0	
Grade							0.87
I	69	12.9	13	12.1	56	13.1	
II	311	58.0	64	59.8	247	57.6	
III	84	15.7	18	16.8	66	15.4	
Unknown	72	13.4	12	11.2	60	14.0	
Stage							<0.01
I	89	16.6	25	23.4	64	14.9	
II	212	39.6	53	49.5	159	37.1	
III	115	21.5	24	22.4	91	21.2	
IV	85	15.9	2	1.9	83	19.3	
Unknown	35	6.5	3	2.8	32	7.5	
Chemotherapy							0.25
Yes	68	12.7	10	9.3	58	13.5	
No	468	87.3	97	90.7	371	86.5	
Radiotherapy							0.86
Yes	9	1.7	2	1.9	7	1.6	
No	527	98.3	105	98.1	422	98.4	
Surgery							<0.01
Yes	463	86.4	105	98.1	358	83.4	
No	73	13.6	2	1.9	71	16.6	
Pulmonary							0.15
Yes	67	12.5	9	8.4	58	13.5	
No	469	87.5	98	91.6	371	86.5	
Cardiovascular							0.35
Yes	244	45.5	53	49.5	191	44.5	
No	292	54.5	54	50.5	238	55.5	
Diabetes							0.64
Yes	62	11.6	11	10.3	51	11.9	
No	474	88.4	96	89.7	378	88.1	
Comorbidity							0.86
0-1	439	81.9	87	81.3	352	82.1	
2+	97	18.1	20	18.7	77	17.9	

[a] SD=Standard Deviation

with stage I colon cancer. Most of the patients did not receive chemotherapy (87%) or radiotherapy (98%). This was similar in both groups. Aspirin users more frequently underwent surgery compared to non-users. There were no differences in co-morbidities between the two groups.

Survival with time-varying covariate

Between 1998 and 2007, 339 patients (63.2%) died during follow-up, and 197 patients were still alive in 2007. For all patients with colon cancer, aspirin use after the diagnosis was associated with a significant reduction in overall mortality (Rate Ratio (RR) 0.51 (95% CI 0.38-0.70 $p<0.001$)). Multivariable analysis revealed that aspirin use was also associated with better survival when adjusted for sex, stage, age, adjuvant chemotherapy, co-morbidity, incidence year, surgery and grade (adjusted RR 0.59 (95% CI 0.44-0.81, $p=0.001$)). Figure 1 shows the OS curve for aspirin users and non-users. Stratification for various factors, as shown in Figure 2, revealed survival gain was present in all strata. The greatest association between aspirin use and survival was in patients with higher disease stage and grade, and in patients who did not receive chemotherapy (adjusted RR for no chemotherapy: 0.71, 95% CI 0.64-0.79, $p<0.001$). Because older patients are frequently known to have co-morbidities we also stratified for this possible confounder. Again, the association between aspirin use and survival persisted in patients with diabetes (adjusted RR 0.53, 95% CI 0.32-0.86, $p=0.01$), cardiovascular disease (adjusted RR 0.69, 95% CI 0.59-0.79, $p<0.001$), no cardiovascular disease (adjusted RR 0.77, 95% CI 0.66-0.89, $p=0.001$) and absence of pulmonary disease (adjusted RR 0.72, 95% CI 0.64-0.80, $p<0.001$).

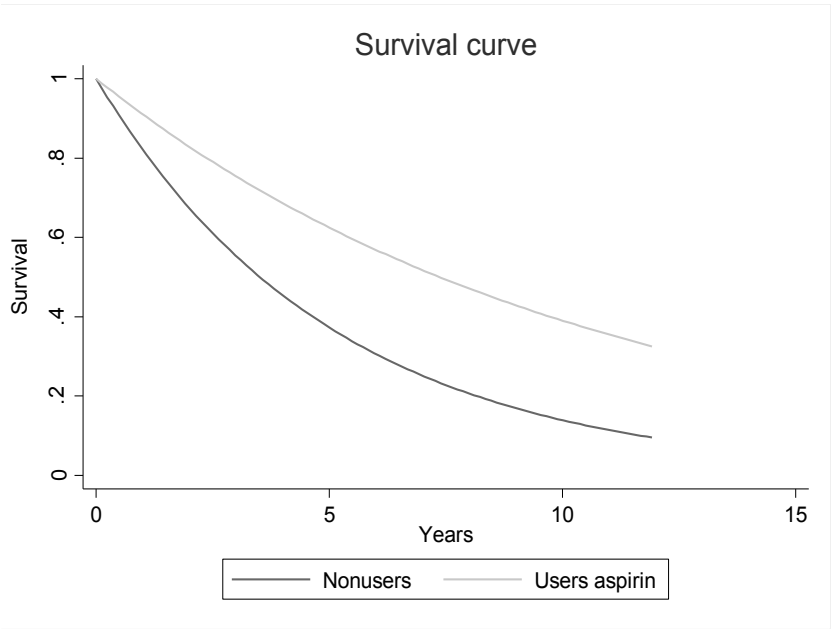


Figure 1: Survival Curve for Overall Survival in Older Colon Cancer Patients According to Use of Aspirin.

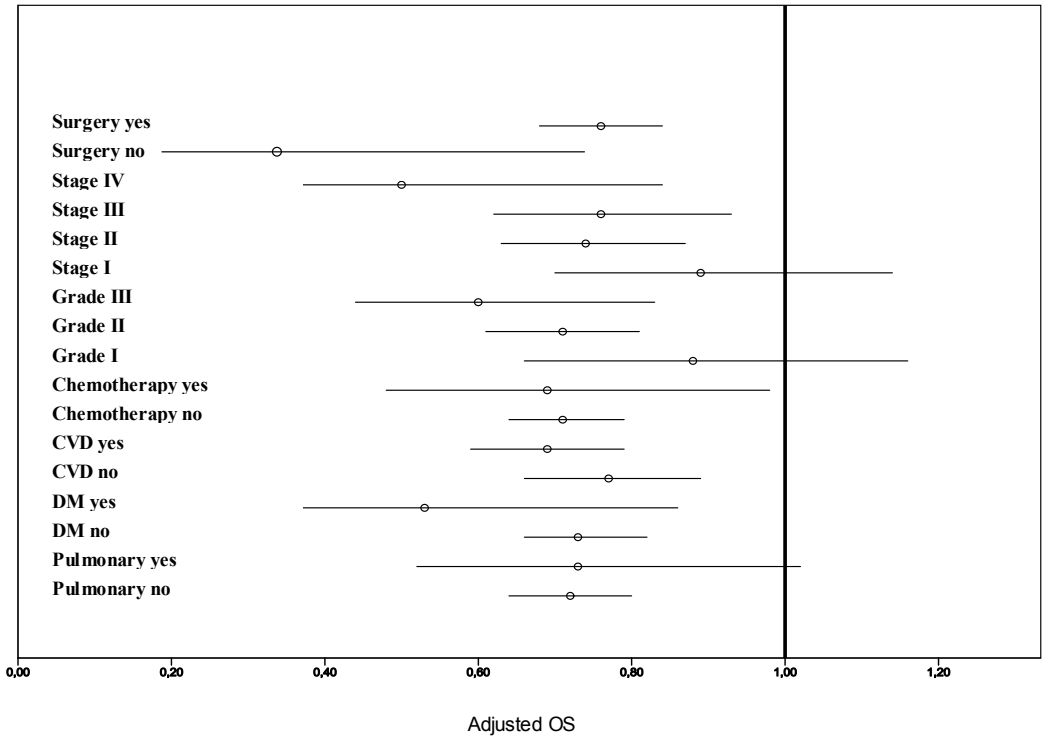


Figure 2: Adjusted Rate Ratio (RR) with 95% Confidence Interval (CI) for Aspirin Use for Older Colon Cancer Patients Stratified for Sex, Stage, Age, Adjuvant chemotherapy, Co-morbidity, Incidence year, Surgery and Grade.
CVD, Cardiovascular disease; DM, diabetes mellitus

DISCUSSION

Here we report an independent strong association of improved survival in older patients who used aspirin after colon cancer diagnosis. This effect also persisted after adjusting for several confounders and was present in most strata of colon cancer.

Since 1968, it has been suggested that aspirin could be a possible preventive agent for colorectal cancer [16]. Only recently, aspirin has been mentioned as a possible adjuvant agent for colorectal cancer [11]. Our study implicates that aspirin could be an effective adjuvant agent in the treatment of colorectal cancer, especially in older, chemo-naive colon cancer patients, as aspirin use was associated with a clinically and statistically significant increase in overall survival. To our knowledge, this is the first report that focuses on older colon cancer patients specifically.

Our results are consistent with results by Chan *et al.*, who found an improved OS of 0.79 (95% CI 0.65-0.97) for regular aspirin users compared to non-users in a cohort study of 1279 patients diagnosed with stage I-III colorectal cancer [11]. In our study as-

pirin users had a RR of 0.51 (95% CI 0.38-0.70, p<0.001) for OS. A major strength of this investigation is the use of a time-dependent covariate in the survival analyses and the large number of patients enrolled in the PHARMO database, which gave us the unique opportunity to assess older colon cancer patients specifically. By using two validated databases we have avoided the possibility of recall bias, which will be more likely with the use of questionnaires to assess aspirin use.

Our results underscore the findings found in cardiovascular prevention trials, where long-term aspirin use was associated with fewer deaths due to cancer. Hazard ratios in these studies ranged from 0.63-0.85, which correspond with our findings, in favor of aspirin use to reduce cancer death. Benefit increased with treatment duration and was consistent across the various populations included in these studies [9;10]. Nevertheless, we assessed aspirin as adjuvant treatment, starting after diagnosis of colon cancer, whereas these studies investigated aspirin use in the preventive setting, including aspirin use before diagnosis. Our results suggest that aspirin use after cancer diagnosis is associated with a survival advantage, when compared to aspirin use before diagnosis. Also, the slightly greater survival advantage for older aspirin users in our cohort might be explained by the undertreatment of these elderly patients with adjuvant chemotherapy, while younger patients receive chemotherapy more often, with good results. Therefore, the absolute effect of aspirin could be higher in older colon cancer patients who, without chemotherapy, have a higher a-priori chance of developing metastases. This is also reflected in the larger effect of aspirin on survival in older colon cancer patients without chemotherapy (HR 0.71) and the previously published data where the largest survival gain of aspirin use after diagnosis was found in older colon cancer patients, when compared to other age categories [1]. Furthermore, the expression of COX-2 may increase with older age and this could be the reason for the larger effect of aspirin on survival in older colon cancer patients [17].

In recent studies, in which prediagnosis NSAID use and survival following colorectal cancer diagnosis was evaluated, a higher reduction in colorectal cancer mortality risk after diagnosis by aspirin use was found compared to overall NSAID use [1;18;19]. These results, along with results of our study, suggest that aspirin and not overall NSAID use, which was mostly used in all previous studies[12;18], may be an important agent in improving survival in colon cancer patients.

Most studies evaluated the use of aspirin or other non-steroidal anti-inflammatory agents before diagnosis. Our study established a longer survival in aspirin users, when started after cancer diagnosis and surgery. Also, due to the large number of patients in the total cohort (4481 colorectal cancer patients) we were able to perform an analysis on older chemo-naive colon cancer patients. Although currently only hypothesis generating, our results suggest that aspirin use as an adjuvant therapy for colon cancer treatment is a clinically relevant option, especially in older adults.

Our study has limitations inherent to observational studies. First, aspirin use was not randomized, so it is possible that patients took aspirin for cancer prevention purposes. However, in the Netherlands, low dose aspirin (80 mg) is exclusively prescribed for cardiovascular risk management, and cannot be purchased 'over the counter.' Second, our data is limited to prescribed drugs. Therefore it is not possible to obtain information regarding to aspirin use or other NSAIDs at home. Third, the improved prognosis could also be explained by the reduced number of cardiac events. However, a meta-analysis for aspirin in the primary and secondary prevention of vascular disease showed a survival gain around 5% for aspirin users [20]. This minimal gain in survival cannot explain the larger survival gain associated with aspirin use in our study. Finally, there were differences in baseline characteristics of patients included in our investigation. Aspirin users more frequently underwent surgery compared to non-users, had lower stage disease and were slightly younger. However, even after adjustment for these confounders and after stratification, the effect of aspirin persisted (Figure 2). More importantly, this longer survival in aspirin users in our study and in other observational studies was consistent with the findings in randomized trials.[21] Nevertheless, residual confounding may still be present. This could only be resolved in a randomized clinical trial, one of which has already been started in Asia (ASCOLT NCT 00565708) and two trials are in preparation in Europe [22].

The exact mechanism by which aspirin exerts its activity is not completely understood. It is likely that the anti-inflammatory and chemopreventive effects of aspirin are mediated through direct inhibition of COX-1 and COX-2 [13;23;24]. Approximately 70% of colorectal tumors express COX-2 [12]. COX-2 plays an important role in colorectal carcinogenesis, invasion, angiogenesis and metastasis. Several studies have shown that this COX-2 effect can be reversed by selective COX-2 inhibitors [13]. Chan *et al.* found a much lower risk of colorectal cancer-specific and overall mortality with tumors that over express COX-2 [11]. Elevated COX-2 expression was found to be associated with tumor metastases, and multiple studies demonstrated COX-2 overexpression as a negative prognostic factor in colorectal cancer [25-27]. Also, studies have linked the COX enzyme-mediated mechanisms to the ability of tumors to initiate vascularization [28] and angiogenesis [29], probably through the production of prostaglandin by COX-2 [30]. This prostaglandin pathway may also be responsible for the regulation of apoptosis [31], and evading apoptosis is one of the key hallmarks of cancer [32]. By using aspirin, a COX-2 inhibitor, the effects of COX-2 on tumor progression can ultimately be altered in a positive way.

This is the first study focusing specifically on older colon cancer patients. Elderly patients are a frequently overlooked, understudied and often undertreated group of patients. Our findings may have important clinical implications in older adults with colorectal cancer. Demonstration of a significant therapeutic effect of a well-tolerated, inexpensive drug would be a major clinical advancement. In this study, aspirin is impli-

cated as an effective adjuvant agent, increasing overall survival in older colon cancer patients. However, a randomized trial in this age group is necessary to confirm the therapeutic role of aspirin, and is currently being developed in the Netherlands.

REFERENCE LIST

(1) Bastiaannet E, Sampieri K, Dekkers OM et al. Use of Aspirin postdiagnosis improves survival for colon cancer patients. *Br J Cancer* 2012.

(2) Devon KM, Vergara-Fernandez O, Victor JC, McLeod RS. Colorectal cancer surgery in elderly patients: presentation, treatment, and outcomes. *Dis Colon Rectum* 2009;52:1272-1277.

(3) Cunningham D, Atkin W, Lenz HJ et al. Colorectal cancer. *Lancet* 2010;375:1030-1047.

(4) van den Broek CB, Dekker JW, Bastiaannet E et al. The survival gap between middle-aged and elderly colon cancer patients. Time trends in treatment and survival. *Eur J Surg Oncol* 2011;37: 904-912.

(5) Andre T, Boni C, Mounedji-Boudiaf L et al. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. *N Engl J Med* 2004;350:2343-2351.

(6) Jessup JM, Stewart A, Greene FL, Minsky BD. Adjuvant chemotherapy for stage III colon cancer: implications of race/ethnicity, age, and differentiation. *JAMA* 2005;294:2703-2711.

(7) Sargent DJ, Goldberg RM, Jacobson SD et al. A pooled analysis of adjuvant chemotherapy for resected colon cancer in elderly patients. *N Engl J Med* 2001;345:1091-1097.

(8) Burn J, Gerdes AM, Macrae F et al. Long-term effect of aspirin on cancer risk in carriers of hereditary colorectal cancer: an analysis from the CAPP2 randomised controlled trial. *Lancet* 2011;378: 2081-2087.

(9) Rothwell PM, Fowkes FG, Belch JF, Ogawa H, Warlow CP, Meade TW. Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. *Lancet* 2011;377:31-41.

(10) Rothwell PM, Price JF, Fowkes FG et al. Short-term effects of daily aspirin on cancer incidence, mortality, and non-vascular death: analysis of the time course of risks and benefits in 51 randomised controlled trials. *Lancet* 2012.

(11) Chan AT, Ogino S, Fuchs CS. Aspirin use and survival after diagnosis of colorectal cancer. *JAMA* 2009;302:649-658.

(12) Midgley RS, McConkey CC, Johnstone EC et al. Phase III randomized trial assessing rofecoxib in the adjuvant setting of colorectal cancer: final results of the VICTOR trial. *J Clin Oncol* 2010;28: 4575-4580.

(13) Chen WS, Wei SJ, Liu JM, Hsiao M, Kou-Lin J, Yang WK. Tumor invasiveness and liver metastasis of colon cancer cells correlated with cyclooxygenase-2 (COX-2) expression and inhibited by a COX-2-selective inhibitor, etodolac. *Int J Cancer* 2001;91:894-899.

(14) Yao M, Zhou W, Sangha S et al. Effects of nonselective cyclooxygenase inhibition with low-dose ibuprofen on tumor growth, angiogenesis, metastasis, and survival in a mouse model of colorectal cancer. *Clin Cancer Res* 2005;11:1618-1628.

(15) van Herk-Sukel MP, van de Poll-Franse LV, Lemmens VE et al. New opportunities for drug outcomes research in cancer patients: the linkage of the Eindhoven Cancer Registry and the PHARMO Record Linkage System. *Eur J Cancer* 2010;46:395-404.

(16) Gasic GJ, Gasic TB, Stewart CC. Antimetastatic effects associated with platelet reduction. *Proc Natl Acad Sci U S A* 1968;61:46-52.

(17) Siironen P, Ristimaki A, Nordling S, Louhimo J, Haapiainen R, Haglund C. Expression of COX-2 is increased with age in papillary thyroid cancer. *Histopathology* 2004;44:490-497.

(18) Coghill AE, Newcomb PA, Campbell PT et al. Prediagnostic non-steroidal anti-inflammatory drug use and survival after diagnosis of colorectal cancer. *Gut* 2011;60:491-498.

(19) Din FV, Theodoratou E, Farrington SM et al. Effect of aspirin and NSAIDs on risk and survival from colorectal cancer. *Gut* 2010;59:1670-1679.
(20) Baigent C, Blackwell L, Collins R et al. Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. *Lancet* 2009;373:1849-1860.
(21) Algra AM, Rothwell PM. Effects of regular aspirin on long-term cancer incidence and metastasis: a systematic comparison of evidence from observational studies versus randomised trials. *Lancet Oncol* 2012.
(22) Ali R, Toh HC, Chia WK. The utility of Aspirin in Dukes C and High Risk Dukes B Colorectal cancer—the ASCOLT study: study protocol for a randomized controlled trial. *Trials* 2011;12:261.
(23) Brown JR, DuBois RN. COX-2: a molecular target for colorectal cancer prevention. *J Clin Oncol* 2005;23:2840-2855.
(24) Wang D, DuBois RN. The role of COX-2 in intestinal inflammation and colorectal cancer. *Oncogene* 2010;29:781-788.
(25) Greenhough A, Smartt HJ, Moore AE et al. The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. *Carcinogenesis* 2009;30:377-386.
(26) Ogino S, Kirkner GJ, Nosho K et al. Cyclooxygenase-2 expression is an independent predictor of poor prognosis in colon cancer. *Clin Cancer Res* 2008;14:8221-8227.
(27) Rodriguez-Moranta F, Castells A. Mechanisms of colon cancer prevention with and beyond COX-2 inhibition. *Curr Top Med Chem* 2005;5:505-516.
(28) Masunaga R, Kohno H, Dhar DK et al. Cyclooxygenase-2 expression correlates with tumor neovascularization and prognosis in human colorectal carcinoma patients. *Clin Cancer Res* 2000;6:4064-4068.
(29) Buchanan FG, Chang W, Sheng H, Shao J, Morrow JD, DuBois RN. Up-regulation of the enzymes involved in prostacyclin synthesis via Ras induces vascular endothelial growth factor. *Gastroenterology* 2004;127:1391-1400.
(30) Cha YI, DuBois RN. NSAIDs and cancer prevention: targets downstream of COX-2. *Annu Rev Med* 2007;58:239-252.
(31) Chan TA. Nonsteroidal anti-inflammatory drugs, apoptosis, and colon-cancer chemoprevention. *Lancet Oncol* 2002;3:166-174.
(32) Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646-674.

CHAPTER 7

Expression of HLA Class I, Aspirin Use and Survival after a diagnosis of Colon Cancer

Marlies S. Reimers, Esther Bastiaannet, Ruth E. Langley, Ronald van Eijk, Ronald L.P. van Vlierberghe, Valery E.P. Lemmens, Myrthe P.P. van Herk-Sukel, Tom van Wezel, Riccardo Fodde, Peter J.K. Kuppen, Hans Morreau, Cornelis J.H. van de Velde, Gerrit Jan Liefers

JAMA Internal Medicine, 2014 May 1;174(5):732-9

ABSTRACT

Background

Use of aspirin (which inhibits platelet function) after a colon cancer diagnosis is associated with improved overall survival. Identifying predictive biomarkers of this effect could individualize therapy and decrease toxicity. Platelets are thought to protect circulating tumor cells from natural killer cells which preferentially eliminate targets with low or absent human leukocyte antigen (HLA) class I expression. We hypothesized that the survival benefit associated with low dose aspirin use after a diagnosis of colorectal cancer might depend upon HLA class I expression.

Methods

A cohort study with tumor blocks from 999 colon cancer patients (surgically resected between 2002 and 2008), analyzed for HLA class I and PTGS2 expression using a Tissue Micro Array (TMA). *PIK3CA* mutation analysis was also performed. Aspirin use post-diagnosis was obtained from a prescription database. Parametric survival models with exponential (Poisson) distribution were used to model overall survival.

Results

The overall survival benefit associated with aspirin use after a diagnosis of colon cancer had an adjusted Rate Ratio (RR) of 0.53 (95% CI 0.38-0.74, $p<0.001$) when tumors expressed HLA class I compared to a RR of 1.03 (95% CI 0.66-1.61 $p=0.9$) when HLA expression was lost. The benefit of aspirin was similar for tumors with strong PTGS2 expression (RR 0.68 95% CI 0.48-0.97, $p=0.03$), weak expression (RR 0.59 95% CI 0.38-0.97, $p=0.02$), and wild-type *PIK3CA* tumors (RR 0.55, 95% CI 0.40-0.75, $p<0.001$). With mutated *PIK3CA* tumors a non-significant trend was observed (RR 0.73 (95% CI 0.33-1.63, $p=0.4$).

Conclusions

Aspirin use after colon cancer diagnosis was associated with improved survival if tumors expressed HLA class I contrary to the original hypothesis. Increased PTGS2 expression or the presence of mutated *PIK3CA* did not predict benefit from aspirin. HLA class I might serve as a predictive biomarker for adjuvant aspirin therapy in colon cancer.

INTRODUCTION

There is a significant body of preclinical, epidemiological and randomized data demonstrating that aspirin has anti-cancer effects [1-7]. Several studies have shown that aspirin use after a diagnosis of colorectal cancer improves colorectal cancer specific and overall survival [2;4;8-10]. Randomized trials designed to assess the cardiovascular benefits of aspirin demonstrate that allocation to aspirin reduces the risk of distant metastasis when cancer is diagnosed (hazard ratio (HR) 0.69 (95% CI 0.5–0.95) p=0.02) and on subsequent follow-up in patients without metastasis at diagnosis (HR 0.45 (95% CI 0.28–0.72) p=0.0009), with the largest effects seen for colorectal cancer (HR at diagnosis 0.36 (95% CI 0.18–0.74) p=0.005 and at follow-up HR 0.26 (95% CI 0.11–0.57) p=0.0008) [7]. Although questions remain about the optimal dose and duration of aspirin use, its efficacy in prediagnostic users and the localization of tumors most likely to benefit, the data suggest that aspirin is a potential adjuvant therapy to prevent distant metastasis in colorectal cancer, and possibly other tumors.

The precise biological mechanisms underlying the anti-cancer effects are unknown. PTGS2 (prostaglandin-endoperoxide synthase 2, also known as cyclo-oxygenase -2) overexpression has been associated with a poor prognosis in colorectal cancer [11;12]. Aspirin inhibits PTGS, at low doses (75-300 mg once daily), given the short half-life of around 30 minutes, this effect is manifest as a permanent inhibition of PTGS1 in the anucleate platelet, which is unable to resynthesize the enzyme. Higher and more frequent dosing for example 600 mg qds would be required to constantly inhibit PTGS2 in systemic tissues [13-16]. Despite this, data from two observational cohorts (the Nurses' Health Study (NHS) and Health Professionals Follow-Up Study (HPFS)), have indicated that the survival benefits of regular low-dose aspirin use after a diagnosis of colorectal cancer are associated with the molecular characteristics of the tumor particularly mutations in the gene *PIK3CA* (a component of the PTGS2 pathway) with a multivariable HR for aspirin users compared to non-users in tumors with mutated *PIK3CA* of 0.18 (95% CI 0.06–0.61; P <0.001) for cancer death and 0.54 (95% CI 0.31–0.94; p = 0.01) for death from any cause [17].

The metastatic potential of cancer cells that are shed into the bloodstream can be modified by environmental conditions, including platelets and bone marrow-derived cells in the vasculature [18]. Platelets are thought to protect disseminating tumor cells from natural killer (NK) cells which preferentially recognize and eliminate cells with low or absent expression of HLA class I [19]. We hypothesized that the survival benefit associated with low dose aspirin use after a cancer diagnosis would be associated with tumors that have low or absent HLA class I expression. We analyzed tumors from a cohort where we had previously shown an association between overall survival (OS) and low-dose aspirin use after diagnosis (adjusted HR 0.65 (95% CI 0.50-0.84; p=0.001) with an even

larger effect in older colon cancer patients ((>70 years) adjusted HR 0.59 (95% CI = 0.44-0.81, $p = 0.001$))[8;10] for HLA class I and PTGS2 expression, and *PIK3CA* mutations.

METHODS

Study cohort

The Eindhoven Cancer Registry was initially used to identify patients diagnosed with colorectal cancer and linked to data on aspirin use from the PHARMO database network (PHARMO, Netherlands). As previously reported, compared with non-users, aspirin initiated or continued after diagnosis was associated with improved survival for colon cancer patients but not rectal cancer patients [8]. Paraffin-embedded tissue blocks were retrieved from 1026 colon cancer patients who had a surgical resection between 2002 and 2008. For this study, 27 patients with more than one colon tumor at the time of diagnosis were excluded thus n=999. There were no significant demographic differences between the patients included in this study and the whole colon cancer cohort in the registry (n= 3586) (Supplementary Table I).

TMA production and immuno-histochemistry

Three 1.0 mm diameter cores were obtained from formalin-fixed paraffin-embedded (FFPE) tumor blocks using haematoxylin and eosin stained sections for tumor identification (with a qualified pathologist confirming the identification of the tumor) and transferred into a receiver paraffin block using the TMA Master (3D Histech, Budapest, Hungary). Immuno-histochemical staining was performed on 4 µm sections, cut from each receiver block and mounted on glass. For each primary antibody, all slides were stained simultaneously to avoid inter-assay variation.

Immuno-histochemical analyses to detect HLA class I expression with mouse monoclonal antibodies HCA2 and HC10 using diaminobenzidine solution (DAB+) (DAKO, Glostrup, Denmark) for visualization of the antibodies, were performed by two independent observers, M.R and R.V, as previously described [20], with good inter-observer agreement (kappa value of 0.5-0.7). The mouse monoclonal antibodies HCA2 and HC10 used recognize the heavy chains of HLA Class I. Their reactivity spectrum has been described in detail before [18]. HLA class I expression status was determined according to the International HLA and Immunogenetics Workshop [21], with tumor cell HLA class I status defined as follows: loss <5% expressing both HCA2 or HC10 or <5% expressing either of the markers and expression as $\geq$ 5% expressing either marker. Normal epithelial, stromal or lymphoid cells served as positive internal controls. PTGS2 expression was analyzed automatically with a double staining to separately visualize stromal cells (using DAB+ for visualization of anti-collagen I, anti-collagen VI and elastin (all polyclonal rabbit antibod-

ies obtained from AbCam)) and positive tumor cells (with the monoclonal mouse antibody anti-PTGS2 (Cayman Chemical Co., Ann Arbor, MI, USA)), using Vector Blue ((Vector Laboratories, Burlingame, USA) for visualization of the PTGS2 antibody. Slides were scanned with the Panoramic Midi scanner (3D-Histech, Hungary) and PTGS2 expression was assessed using the criteria proposed by Buskens *et al.* [22] using AxioVision 4.6 (Zeiss, Jena, Germany), and comparable to the scoring method used by Chan *et al.* [2].

Microsatellite stability status was determined by immuno-histochemical analyses as previously described [23]. In short, four antibodies directed against MutL homolog21 (*MLH1,* clone ES05, DAKO Cytomation, Glostrup, Denmark), MutS homolog 2 (*MSH2,* clone g219-1129, BD Biosciences, Franklin Lakes, USA), MutS homolog 6 (*MSH6,* clone EPR3945, Epitomics, Burlingame, USA) and postmeiotic segregation of *Saccharomyces cerevisia* 2 (*PMS2,* clone A16-4, BD Biosciences, Franklin Lakes, USA) were used. The

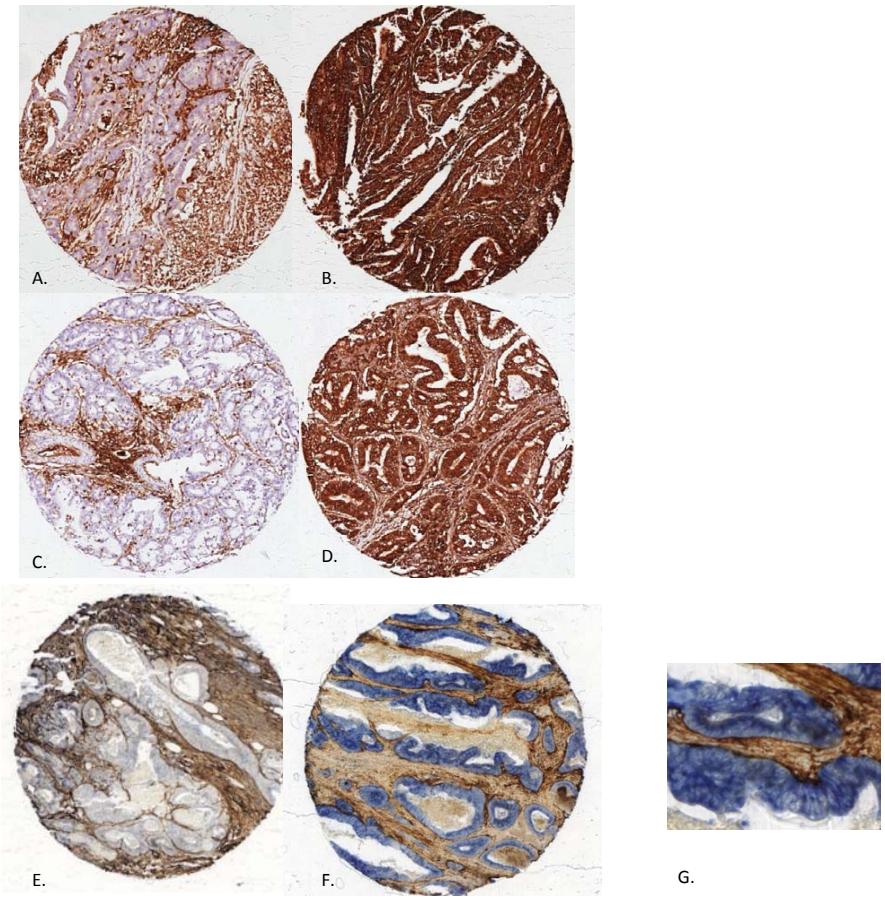


Figure 1: Representative images of HCA2, HC10 and PTGS2 staining in colon cancer.
Representative images of immunohistochemical stainings for HLA Class I expression (HCA2 and HC10) and PTGS2 performed according to standard protocols (details in Material and Methods).(A) HC10-negative tumor (B) HC10-positive tumor (C) HCA2- negative tumor (D) HCA2- positive tumor (E) Tumor with weak PTGS2 expression (F) Tumor with strong PTGS2 expression, with a magnification in (G).

criteria used to confirm microsatellite instability (MSI) in the tissues are described elsewhere [23;24].

All slides were stained simultaneously to avoid interassay variation. Slides that did undergo the whole immunohistochemical staining procedure but without primary antibodies served as negative controls. The quality of the staining, the scoring method and discrepancies between the two observers were checked by a pathologist (H.M).

Representative images of the immuno-histochemical stainings are shown in Figure 1.

PIK3CA mutation analysis

DNA was extracted from 1 to 2, 2.0 mm diameter and variable length cores taken from 663 of the 999 blocks randomly chosen, with a ratio 1:2 for aspirin user: non-user, using a fully automated system (Tissue Preparation System with VERSANT Tissue Preparation Reagents, Siemens Healthcare Diagnostics, Tarrytown, NY, USA) as described previously [25].

Hydrolysis probes assays were performed for the major known mutations (hotspots) in exon 9, c.1624G>A; *p*.E542K, c.1633G>A; p.E545K and in exon 20 the c.3140A>G; p.H1047R as described before [26]. Hydolysis probe assays were analyzed using qPCR analysis software (CFX manager version 3/0, Bio-Rad). To identify additional non-hotspot mutations, Sanger sequencing was performed on exon 9 and exon 20 of all samples. Mutation detection was performed by two observers independently (M.R and R.E) using DNA variant analysis software (Mutation Surveyor version 4.0.9, Softgenetics, State College, PA, USA). All primers and probes used for the assays can be found in Supplementary Table II.

Statistics

The vital status of patients (alive/dead) was established from medical records or through linkage of cancer registry data with the municipal population registries. Follow-up started 30 days from diagnosis of colorectal cancer (T0), as information on hospital prescriptions was not available, and was continued until last contact date (January 2012) or date of death. Patients who died within 30 days were excluded from the survival analyses (2.4% for colon cancer). Non-users were classified as those who never had a prescription for aspirin or had a prescription for less than 14 days after diagnosis of colon cancer. Users were defined as those who had been given a prescription for aspirin for 14 days or more after a colon cancer diagnosis. The median duration of prescriptions was 30 days and the mean number of prescriptions was 12 (range 1- 220). Non-users were defined from T0 to first use and users from first use to the end of the follow-up in the time-dependent exposure survival analysis.

As the data was split in two episodes for users (multiple ID rows for one patient), we were not able to model a Cox proportional hazard model and used a parametric

survival model with an exponential (Poisson) distribution after the data was declared as survival-time data (stset) and split at the time to first prescription.

Adjustments for potential confounders were made for sex, age (continuous), stage (pathological stage and clinical stage if pathological stage was unknown), adjuvant chemotherapy (yes/no), co-morbidity (yes/no), tumor grade and year of diagnosis. Stratified analyses were performed for HLA class I expression, weak or strong PTGS2 expression and for wild-type *PIK3CA* / *PIK3CA* mutation.

RESULTS

Aspirin use, survival and tumor HLA class I expression

Of the 999,18.2% (182/999) were defined as aspirin users and there had been 465 deaths recorded until January 2012. There were 396 deaths in 817 nonusers (48.5%) and 69 deaths in 182 aspirin users (37.9%) after diagnosis. In this cohort, aspirin use after diagnosis was associated with an improved OS (RR 0.64, 95% CI 0.49-0.83, p=0.001), when compared to nonusers.

36/999 tumors could not be analyzed for HLA class I expression due to staining artefacts or loss of material. Table I summarizes the clinical characteristics of the patients presented by HLA class I expression and according to aspirin use/non-use after diagnosis. Loss of HLA class I expression was found in 33.2% (320/963) and expression in 66.8% (643/963), in accord with results from previous studies [27;28]. Aspirin use was similar in both groups, loss of HLA 18% (57/320) and expression of HLA class I 19% (122/643),

Table I: Baseline Characteristics of the Colon Cancer Patients according to Tumor HLA Class I Expression and Use of Aspirin after Diagnosis

	All patients (N=999)	HLA Loss (N=320)			HLA Expression (N=643)		
		No aspirin	Aspirin	*p*-value	No aspirin	Aspirin	*p*-value
Sex							
Male	505 (50.6)	121 (46.0)	35 (61.4)	0.04	260 (49.9)	78 (63.9)	0.005
Female	494 (49.4)	142 (54.0)	22 (38.6)		261 (50.1)	44 (36.1)	
Age							
<65	342 (34.2)	110 (41.8)	8 (14.0)	<0.001	188 (36.1)	23 (18.8)	0.001
66-74	304 (30.4)	66 (25.1)	26 (45.6)		155 (29.7)	45 (36.9)	
75 and older	353 (35.4)	87 (33.1)	23 (40.4)		178 (34.2)	54 (44.3)	
Year of diagnosis							
2002-2004	451 (45.2)	102 (38.8)	27 (47.4)	0.2	232 (44.5)	65 (53.3)	0.08
2005-2007	548 (54.8)	161 (61.2)	30 (52.6)		289 (55.5)	57 (46.7)	

Table I: Baseline Characteristics of the Colon Cancer Patients according to Tumor HLA Class I Expression and Use of Aspirin after Diagnosis *(Continued)*

	All patients (N=999)	HLA Loss (N=320)			HLA Expression (N=643)		
		No aspirin	Aspirin	*p*-value	No aspirin	Aspirin	*p*-value
Disease stage							
I	138 (13.8)	24 (9.1)	5 (8.8)	0.2	71 (13.6)	33 (27.0)	<0.001
II	402 (40.2)	108 (41.1)	30 (52.6)		210 (40.3)	39 (32.0)	
III	287 (28.7)	77 (29.3)	17 (29.8)		142 (27.3)	40 (32.8)	
IV	169 (16.9)	54 (20.5)	5 (8.8)		95 (18.2)	10 (8.2)	
Unknown	3 (0.3)				3 (0.6)		
Comorbidity							
No	443 (44.3)	138 (52.5)	14 (24.6)	<0.001	253 (48.6)	25 (20.5)	<0.001
Yes	556 (55.7)	125 (47.5)	43 (75.4)		268 (51.4)	97 (79.5)	
Microsatellite status							
MSI	90 (9.0)	28 (10.7)	11 (19.3)	0.2	38 (7.3)	8 (6.6)	0.9
MSS	870 (87.1)	227 (86.3)	45 (78.9)		472 (90.6)	112 (91.8)	
Unknown	39 (3.9)	8 (3.0)	1 (1.8)		11 (2.1)	2 (1.6)	

Overall aspirin use: 182 patients (18.2%).

Table II: Rate Ratio for Death (Time-Dependent Analysis Overall Survival), According to Tumor HLA Class I Expression, PTGS2 Expression and *PIK3CA* Mutation Status and Use or Nonuse of Aspirin after Diagnosis

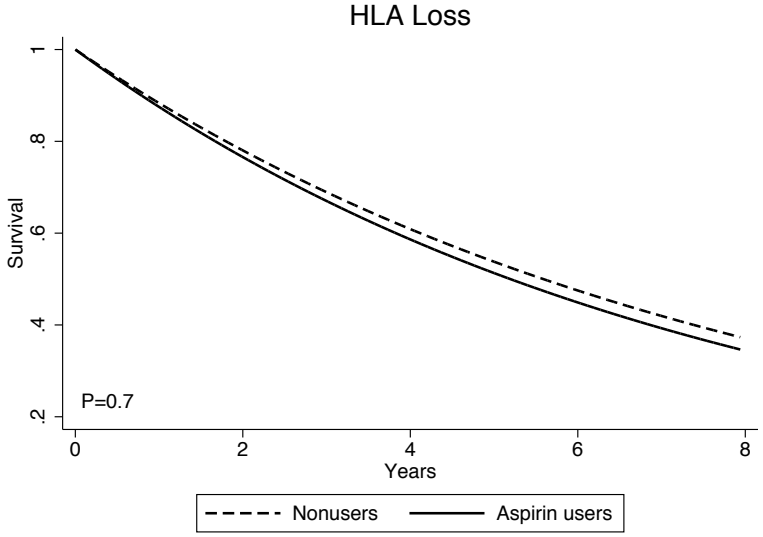
	Patients	Deaths	Univariate RR (95%CI)	*p*-value	Adjusted RR* (95%CI)	*p*-value	*p*-value (interaction)**
HLA Class I							0.007
Loss							
No aspirin use	263	123	1.00 (reference)	0.7	1.00 (reference)	0.9	
Aspirin use	57	26	1.08 (0.70-1.64)		1.03 (0.66-1.61)		
Expression							
No aspirin use	521	257	1.00 (reference)	0.003	1.00 (reference)	<0.001	
Aspirin use	122	42	0.61 (0.44-0.85)		0.53 (0.38-0.74)		
PTGS2							0.12
Low							
No aspirin use	360	190	1.00 (reference)	0.05	1.00 (reference)	0.02	
Aspirin use	66	25	0.66 (0.44-1.01)		0.59 (0.38-0.91)		
High							
No aspirin use	434	192	1.00 (reference)	0.2	1.00 (reference)	0.03	
Aspirin use	114	42	0.80 (0.57-1.12)		0.68 (0.48-0.97)		
PIK3CA							0.004
Wild-type							
No aspirin use	384	200	1.00 (reference)	0.007	1.00 (reference)	<0.001	
Aspirin use	147	55	0.66 (0.49-0.89)		0.55 (0.40-0.75)		
Mutation							
No aspirin use	73	34	1.00 (reference)	0.3	1.00 (reference)	0.4	
Aspirin use	27	9	0.70 (0.34-1.46)		0.73 (0.33-1.63)		

*Aspirin use = use of aspirin after diagnosis. *Adjusted for sex, age, comorbidity, year of incidence, histological grade, stage and chemotherapy. ** Interaction between the markers and aspirin use status (user/nonuser).*

though aspirin users were older and more likely to have co-morbidity. In the HLA class I expression group, there were more lower stage tumors in the aspirin users compared to non-users (p<0.001).

The effect of HLA class I expression status on the survival benefit associated with post-diagnosis aspirin use was examined (Table II and Figure 2). For patients whose tumors expressed HLA class I, aspirin use after diagnosis was associated with a significantly

A



B

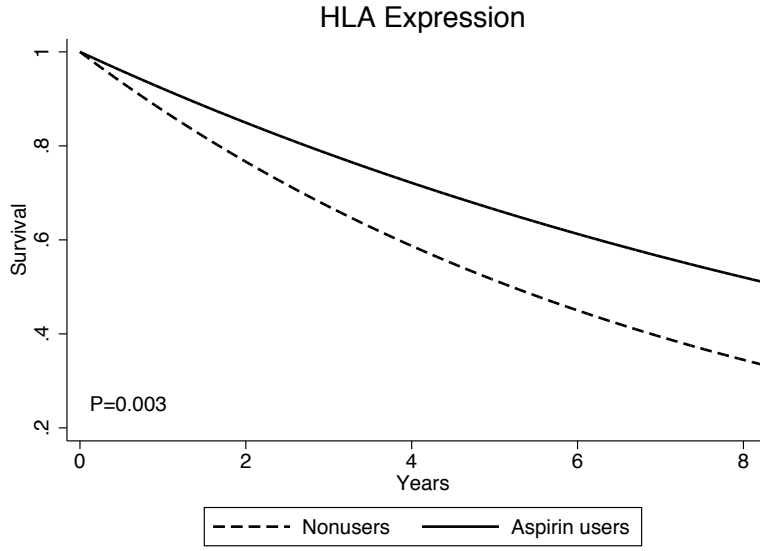


Figure 2: Overall Survival Curves Aspirin Use versus No Aspirin Use Stratified by HLA Class I.
Survival curves for overall survival in colon cancer patients, according to aspirin use after diagnosis or nonuse of aspirin after diagnosis and HLA Class I expression. Above: Overall survival among colon cancer patients with loss of HLA class I in their tumor sections. Below: Survival among colon cancer patients with expression of HLA Class I in their tumor sections.

longer OS, RR 0.61 (95% CI 0.44-0.85, p=0.003), and when adjusted for potential confounders, this effect remained with an adjusted RR of 0.53 (95% CI 0.38-0.74, p<0.001). In contrast, for patients whose tumors had loss of HLA class I expression, aspirin use after diagnosis was not associated with a survival benefit (Adjusted RR 1.03 (95% CI 0.66-1.61, p=0.9).

Aspirin use, survival and tumor PTGS2 expression and *PIK3CA* mutations

25/999 samples could not be analyzed for PTGS2 expression due to staining artefacts or loss of material. Weak expression of PTGS2 was seen in 43.7% (426/974) of samples, and strong PTGS2 expression in 56.3% (548/974) in accord with the literature [2;29]. Use of aspirin after diagnosis was significantly associated with a survival benefit, both when tumors showed weak PTGS2 expression (Adjusted RR 0.59, 95% CI 0.38-0.91, p=0.02) and with strong PTGS2 expression (Adjusted RR 0.68, 95% CI 0.48-0.97, p=0.03) (Table II).

DNA was extracted from 663 tumor blocks and *PIK3CA* mutation status (wild-type/mutation) was established in 95% (631/663) of the samples. Baseline characteristics among participants with colon cancer whom we analyzed for *PIK3CA* were largely similar as the baseline characteristics of the PTGS2/HLA class I cohort (mean age at inclusion, 70.38 vs. 69.01 years; male, 54% vs. 51%; stage I, 15% vs. 14%; stage II, 40% vs. 40%; stage III, 29% vs. 29%; stage IV 15% vs. 17%; presence of comorbidity, 60% vs. 56%; adjuvant chemotherapy, 28% vs. 31%; histological grade I, 11% vs. 11%; grade II, 70% vs. 68%; grade III 19% vs. 21%; p>0.091 for all comparisons). A *PIK3CA* mutation was found in 15.8% (100/663), also in accord with what has been found previously [30]. Aspirin use was 27% (27/100) among patients with a mutated *PIK3CA* tumor and 28% (147/531) in patients with a *PIK3CA* wild-type tumor. Aspirin use after a colon cancer diagnosis was significantly associated with a better OS among patients with a wild-type *PIK3CA* tumor (Adjusted RR 0.55, 95% CI 0.40-0.75, p<0.001). In patients with a *PIK3CA* mutation, post-diagnosis aspirin use showed the same trend (though non-significant) with an adjusted RR of 0.73 (95% CI 0.33-1.63, p=0.4). The small number of deaths (9) among patients with mutated *PIK3CA* tumors precludes robust statistical assessment (Table II).

DISCUSSION

We found that the survival benefit associated with low-dose aspirin use after a diagnosis of colon cancer was significantly associated with HLA class I positive tumors. In contrast, in patients whose tumors had lost their HLA expression, aspirin use did not change outcome. PTGS2 expression and *PIK3CA* mutation analysis could not identify patients with a high likelihood of benefit from aspirin in contrast to previous studies [2;17;31].

Currently, the molecular mechanism(s) underlying the anti-cancer effects of aspirin are incompletely understood. Given that the majority of our cohort (> 80%) were diagnosed as stage III or less at the time of diagnosis the predominant effect of aspirin on cancer outcomes is likely to result from an effect on circulating tumor cells and their ability to develop into metastatic deposits. Natural killer (NK) cells play an important role in tumor immune-surveillance, preferentially eliminating targets with low or absent expression of HLA class I [19]. Adhesion of HLA expressing platelets to tumor cells with absent or low HLA class I expression is thought to result in a "pseudonormal phenotype" and reduced NK mediated lysis [19]. We originally hypothesized that aspirin might inhibit platelet adhesion to tumor cells leaving those with absent or low HLA Class I expression susceptible to immune clearance, however we unexpectedly found that the effect of aspirin is dependent on intact HLA class I expression within the original primary tumor, and assuming that circulating tumor cells retain the same HLA phenotype as the original tumor does not support the hypothesis that the attenuation of metastases by aspirin and possibly other anticoagulants is a result of enhanced NK activity [19].

A possible explanation for this intriguing observation is that HLA expression might be necessary for platelet mediated NF-κB signaling in circulating tumor cells resulting in an epithelial-mesenchymal-like phenotype with enhanced metastatic potential [18]. In this model direct contact of platelets and tumor cells results in secretion of TGF-β and activation of the NF-κB pathway, which, in synergistic action, prime circulating tumor cells for subsequent metastases. In a breast cancer model acquisition of an epithelial-mesenchymal phenotype markedly reduced susceptibility of cancer cells to T-cell mediated immune surveillance in-vitro [32]. Our data would be compatible with the hypothesis that aspirin inhibits platelet-tumor cell signaling (which is dependent upon intact HLA expression) and prevents epithelial-mesenchymal transition in circulating tumor cells, thereby reducing the metastatic potential.

Our data has not confirmed previous reports that the benefits of aspirin after a colorectal cancer diagnosis are associated with strong PTGS2 expression in the original tumor and the presence of mutations in *PIK3CA*, with no benefit for patients whose tumors had wild-type *PIK3CA* [2;17]. Liao *et al.* postulated that by blocking the *PIK3CA* pathway PTGS2 activity decreases, which leads to apoptosis of colon cancer cells and which was in accord with their previous work demonstrating a clinical benefit of aspirin in patients with PTGS2 positive tumors [2]. In a separate study, benefits of aspirin in *PIK3CA* mutated tumors were seen but the correlation with strong expression of PTGS2 expression was not confirmed [31].

Pharmacological data on aspirin indicate that systemic concentrations of aspirin, reached with low-doses, (75-325 mg once daily) are inadequate to permanently acetylate PTGS2, but are optimal for platelet inhibition [13]. It is possible that there may be more than one mechanism of action that accounts for the anti-cancer effects of aspirin. A

direct anti-platelet effect due to PTGS1 inhibition that is responsible for the reduction in metastases and only requires a dose of aspirin that inhibits platelets, and a second mechanism, possibly mediated through platelets again, or perhaps activated with higher or more frequent dosing that inhibits the PTGS2 pathway in systemic tissues and may partly explain the differences between the results of our study and that of Liao *et al*. In breast cancer it has also been reported that PTGS2 expression could not identify a subgroup of patients where aspirin decreased recurrence [33]. Furthermore, in breast cancer low dose aspirin did not influence local recurrence, but was significantly associated with a decrease in metastatic disease [34].

Aspirin use has also been associated with a decreased risk of developing a colorectal tumor with an intact *BRAF* gene but no association between post-diagnosis aspirin use, *BRAF* mutation status and clinical outcome has been found [35]. *BRAF* is a member of RAF-MAPK signaling pathway and involved in the up-regulation of PTGS2 again suggesting aspirin may have differential effects on carcinogenesis and prevention of metastatic spread [35].

Strengths of our study include a more precise definition of regular aspirin use and dose as this information was derived from prescriptions (rather than patient recall), noting also that low-dose aspirin is not available as an over-the-counter medication in the Netherlands, thereby minimizing non-differential misclassification of exposure. Higher dose over-the-counter aspirin use is unknown, which could have biased our results towards the null hypothesis. However, it has been shown that, pharmacy data can give valid associations even though a high proportion (25%) of the drugs are available over-the-counter [36]. Other limitations of our study include the inherent issue that this is non-randomized data, compliance is unknown, and some subgroups contained small numbers of events, although our series is the largest study thus far that has reported on aspirin use in colon cancer patients.

The molecular profiling of tumors for example *KRAS* testing in colorectal cancer and HER-2 testing in breast cancer has become standard clinical practice and the basis of therapeutic decisions. If the association of HLA expression and benefit from aspirin is confirmed in other datasets it could be used in clinical practice, where, our data may have important clinical implications for both the dose and timing of aspirin as an anti-cancer agent. First, low dose daily aspirin may suffice as an anti-metastatic therapy in early stage cancer patients. Second, as circulating tumor cells are found in the peri-operative period, it could be argued that aspirin should be commenced as soon as considered clinically appropriate after diagnosis.

In conclusion, we report the novel finding that the survival benefit associated with low-dose aspirin use after diagnosis of colon cancer is dependent on intact HLA class I expression in the original tumor. Randomized trials of the use of aspirin in the adjuvant

setting may provide key information about platelet-tumor interactions and the signaling pathways they elicit.

REFERENCE LIST

(1) Algra AM, Rothwell PM. Effects of regular aspirin on long-term cancer incidence and metastasis: a systematic comparison of evidence from observational studies versus randomised trials. *Lancet Oncol* 2012;13:518-527.

(2) Chan AT, Ogino S, Fuchs CS. Aspirin use and survival after diagnosis of colorectal cancer. *JAMA* 2009;302:649-658.

(3) Langley RE, Burdett S, Tierney JF, Cafferty F, Parmar MK, Venning G. Aspirin and cancer: has aspirin been overlooked as an adjuvant therapy? *Br J Cancer* 2011;105:1107-1113.

(4) McCowan C, Munro AJ, Donnan PT, Steele RJ. Use of aspirin post-diagnosis in a cohort of patients with colorectal cancer and its association with all-cause and colorectal cancer specific mortality. *Eur J Cancer* 2013;49:1049-1057.

(5) Rothwell PM, Wilson M, Elwin CE et al. Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomised trials. *Lancet* 2010;376:1741-1750.

(6) Rothwell PM, Fowkes FG, Belch JF, Ogawa H, Warlow CP, Meade TW. Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. *Lancet* 2011;377:31-41.

(7) Rothwell PM, Wilson M, Price JF, Belch JF, Meade TW, Mehta Z. Effect of daily aspirin on risk of cancer metastasis: a study of incident cancers during randomised controlled trials. *Lancet* 2012; 379:1591-1601.

(8) Bastiaannet E, Sampieri K, Dekkers OM et al. Use of aspirin postdiagnosis improves survival for colon cancer patients. *Br J Cancer* 2012;106:1564-1570.

(9) Chia WK, Ali R, Toh HC. Aspirin as adjuvant therapy for colorectal cancer—reinterpreting paradigms. *Nat Rev Clin Oncol* 2012;9:561-570.

(10) Reimers MS, Bastiaannet E, van Herk-Sukel MP et al. Aspirin use after diagnosis improves survival in older adults with colon cancer: a retrospective cohort study. *J Am Geriatr Soc* 2012;60:2232-2236.

(11) Dong M, Johnson M, Rezaie A et al. Cytoplasmic phospholipase A2 levels correlate with apoptosis in human colon tumorigenesis. *Clin Cancer Res* 2005;11:2265-2271.

(12) Soumaoro LT, Uetake H, Higuchi T, Takagi Y, Enomoto M, Sugihara K. Cyclooxygenase-2 expression: a significant prognostic indicator for patients with colorectal cancer. *Clin Cancer Res* 2004; 10:8465-8471.

(13) Bruno A, Dovizio M, Tacconelli S, Patrignani P. Mechanisms of the antitumoural effects of aspirin in the gastrointestinal tract. *Best Pract Res Clin Gastroenterol* 2012;26:e1-e13.

(14) Dovizio M, Bruno A, Tacconelli S, Patrignani P. Mode of action of aspirin as a chemopreventive agent. *Recent Results Cancer Res* 2013;191:39-65.

(15) FitzGerald GA, Oates JA, Hawiger J et al. Endogenous biosynthesis of prostacyclin and thromboxane and platelet function during chronic administration of aspirin in man. *J Clin Invest* 1983;71: 676-688.

(16) Patrignani P, Filabozzi P, Patrono C. Selective cumulative inhibition of platelet thromboxane production by low-dose aspirin in healthy subjects. *J Clin Invest* 1982;69:1366-1372.

(17) Liao X, Lochhead P, Nishihara R et al. Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. *N Engl J Med* 2012;367:1596-1606.

(18) Labelle M, Begum S, Hynes RO. Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. *Cancer Cell* 2011;20:576-590.

(19) Placke T, Orgel M, Schaller M et al. Platelet-derived MHC class I confers a pseudonormal phenotype to cancer cells that subverts the antitumor reactivity of natural killer immune cells. *Cancer Res* 2012;72:440-448.

(20) de Kruijf EM, van Nes JG, Sajet A et al. The predictive value of HLA class I tumor cell expression and presence of intratumoral Tregs for chemotherapy in patients with early breast cancer. *Clin Cancer Res* 2010;16:1272-1280.

(21) Chew SF, Kanaan C, Tait BD. HLA expression and cancer—14th IHIWS immunohistochemistry quality control exercise exchange results. *Tissue Antigens* 2007;69 Suppl 1:248-251.

(22) Buskens CJ, Van Rees BP, Sivula A et al. Prognostic significance of elevated cyclooxygenase 2 expression in patients with adenocarcinoma of the esophagus. *Gastroenterology* 2002;122:1800-1807.

(23) de Jong AE, van PM, Hendriks Y et al. Microsatellite instability, immunohistochemistry, and additional PMS2 staining in suspected hereditary nonpolyposis colorectal cancer. *Clin Cancer Res* 2004;10:972-980.

(24) Baudhuin LM, Burgart LJ, Leontovich O, Thibodeau SN. Use of microsatellite instability and immunohistochemistry testing for the identification of individuals at risk for Lynch syndrome. *Fam Cancer* 2005;4:255-265.

(25) van Eijk R, Stevens L, Morreau H, van WT. Assessment of a fully automated high-throughput DNA extraction method from formalin-fixed, paraffin-embedded tissue for KRAS, and BRAF somatic mutation analysis. *Exp Mol Pathol* 2013;94:121-125.

(26) van Eijk R, Licht J, Schrumpf M et al. Rapid KRAS, EGFR, BRAF and PIK3CA mutation analysis of fine needle aspirates from non-small-cell lung cancer using allele-specific qPCR. *PLoS One* 2011;6: e17791.

(27) Speetjens FM, de Bruin EC, Morreau H et al. Clinical impact of HLA class I expression in rectal cancer. *Cancer Immunol Immunother* 2008;57:601-609.

(28) Watson NF, Ramage JM, Madjd Z et al. Immunosurveillance is active in colorectal cancer as downregulation but not complete loss of MHC class I expression correlates with a poor prognosis. *Int J Cancer* 2006;118:6-10.

(29) Al-Maghrabi J, Buhmeida A, Emam E et al. Cyclooxygenase-2 expression as a predictor of outcome in colorectal carcinoma. *World J Gastroenterol* 2012;18:1793-1799.

(30) Farina SA, Zeestraten EC, van WT et al. PIK3CA kinase domain mutation identifies a subgroup of stage III colon cancer patients with poor prognosis. *Cell Oncol (Dordr)* 2011;34:523-531.

(31) Domingo E, Church DN, Sieber O et al. Evaluation of PIK3CA Mutation As a Predictor of Benefit From Nonsteroidal Anti-Inflammatory Drug Therapy in Colorectal Cancer. *J Clin Oncol* 2013.

(32) Akalay I, Janji B, Hasmim M et al. Epithelial-to-mesenchymal transition and autophagy induction in breast carcinoma promote escape from T-cell-mediated lysis. *Cancer Res* 2013;73:2418-2427.

(33) Holmes MD, Chen WY, Schnitt SJ et al. COX-2 expression predicts worse breast cancer prognosis and does not modify the association with aspirin. *Breast Cancer Res Treat* 2011;130:657-662.

(34) Jonsson F, Yin L, Lundholm C, Smedby KE, Czene K, Pawitan Y. Low-dose aspirin use and cancer characteristics: a population-based cohort study. *Br J Cancer* 2013.

(35) Nishihara R, Lochhead P, Kuchiba A et al. Aspirin use and risk of colorectal cancer according to BRAF mutation status. *JAMA* 2013;309:2563-2571.

(36) Yood MU, Campbell UB, Rothman KJ et al. Using prescription claims data for drugs available over-the-counter (OTC). *Pharmacoepidemiol Drug Saf* 2007;16:961-968.

SUPPLEMENTARY MATERIAL

Supplementary Table I: Baseline characteristics of the patients included in the population-based registry and the patients included in this study

		Population-based registry (n=3586)	Patients (n=999)	*p*-value
Sex				
	Male	1849 (51.6)	505 (50.6)	0.57
	Female	1737 (48.4)	494 (49.4)	
Age				
	1	1180 (32.9)	342 (34.2)	0.73
	2	1107 (30.9)	304 (30.4)	
	3	1299 (36.2)	353 (35.4)	
Comorbidity				
	No	1627 (45.4)	443 (44.3)	0.56
	Yes	1959 (54.6)	556 (55.7)	
Stage				
	I / II	1878 (52.4)	540 (54.1)	0.69
	III / IV	1541 (43.0)	456 (45.6)	
	Missing	167 (4.6)	3 (0.3)	
Grade				
	1	452 (12.6)	100 (10.0)	0.12
	2	2053 (57.3)	629 (62.9)	
	3	635 (17.7)	193 (19.3)	
	Missing	446 (12.4)	77 (7.7)	
Localization				
	Proximal	1482 (41.3)	422 (42.2)	0.25
	Distal	588 (16.4)	169 (16.9)	
	Sigmoid	1433 (40.0)	395 (39.5)	
	Other	83 (2.3)	13 (1.3)	

Supplementary Table II: primer overview of the primers used for the *PIK3CA* mutation analysis

Assay Name	Primer Name	Primer sequence
p.E542K*	PIK3CA_p.E542K _F	AGCTCAAAGCAATTTCTACACGAGAT
	PIK3CA_p.E542K _R	GCACTTACCTGTGACTCCATAGAAA
p.E545K*	PIK3CA_p.E545K_F	TCAAAGCAATTTCTACACGAGATCCT
	PIK3CA_p.E545K_R	GCACTTACCTGTGACTCCATAGAAA
p.H1047R*	PIK3CA_p.H1047R_F	GCAAGAGGCTTTGGAGTATTTCATG
	PIK3CA_p.H1047R_R	GCTGTTTAATTGTGTGGAAGATCCAA
Exon 9**	PIK3CA_x9_M13F	TGTAAAACGACGGCCAGTGGGAAAATGACAAAGAACAGC
	PIK3CA_x9_M13R	CAGGAAACAGCTATGACCTCCATTTTAGCACTTACCTGTGAC
Exon 20**	PIK3CA_x20_M13F	TGTAAAACGACGGCCAGTCTGAGCAAGAGGCTTTGGAG
	PIK3CA_x20_M13R	CAGGAAACAGCTATGACCCCTATGCAATCGGTCTTTGC
***	PR_M13F	TGTAAAACGACGGCCAGT
***	PR_M13R	CAGGAAACAGCTATGACC

** Hydrolysis probes assays, ** Genomic PCR, *** Sanger sequencing, F=Forward primer, R=Reverse primer.*

Reporter 1 name	Dye	Reporter 1 Sequence	Reporter 2 name	Dye	Reporter 2 Sequence
PIK3CA_p.E542K _V	VIC	CCTCTCTCTGAAATCA	PIK3CA_p.E542K _M	FAM	CCTCTCTCTAAAATCA
PIK3CA_p.E545K_V	VIC	CTCTCTGAAATCACTGAGCAG	PIK3CA_p.E545K_M	FAM	CTCTGAAATCACTAAGCAG
PIK3CA_p.H1047R_V	VIC	CCACCATGATGTGCATC	PIK3CA_p.H1047R_M	FAM	CACCATGACGTGCATC

PART THREE

Precision medicine in colorectal cancer

CHAPTER 8

Biomarkers in precision therapy in colorectal cancer

Marlies S. Reimers, Eliane C.M. Zeestraten, Peter J.K. Kuppen, Gerrit Jan Liefers, Cornelis J.H. van de Velde

Gastroenterology Report, 2013 Nov;1(3):166-83

ABSTRACT

Colorectal cancer (CRC) is the most commonly diagnosed cancer in Europe. Because CRC is also a major cause of cancer-related deaths worldwide, a lot of research has been dedicated to the discovery and development of biomarkers to improve the diagnostic process and to predict treatment outcomes. Up till now only a few biomarkers are recommend by expert panels. The currently used TNM criteria, however, cause substantial under- and overtreatment of CRC patients. Consequently, there is a growing need for new and efficient biomarkers to ensure optimal treatment allocation. The ideal biomarker is one that can easily be introduced in clinical practice, able to identify patients who can be spared from treatment or capable of identifying patients who will benefit from therapy, ultimately resulting in precision medicine in the future. With this review we aimed to provide an overview of a number of frequently studied biomarkers in CRC and at the same time we will emphasize the difficulties and controversies that withhold the clinical introduction of these biomarkers. We will discuss both prognostic and predictive markers of chemotherapy, aspirin therapy as well as overall therapy toxicity. Currently, only mutant KRAS, mutant BRAF, MSI and the Onco*type* DX Colon Cancer Assay are used in clinical practice. Other biomarker studies showed insufficient evidence to introduce these biomarkers in clinical practice. Divergent patient selection criteria, absence of validation studies, and a large number of single biomarker studies are possibly responsible. We therefore advice future studies to focus on combining key markers rather than analyzing only one marker, standardizing study protocols and to validate the results in independent study cohorts followed by prospective clinical trials.

INTRODUCTION

Colorectal cancer (CRC) is the most frequently diagnosed type of cancer in Europe and is one of the major contributors to cancer-related deaths worldwide [1;2]. In 2008, 436.000 new cases of CRC were diagnosed in Europe and was therefore the most frequently diagnosed cancer with 13.6% of all diagnosed cancers [1]. Worldwide, the percentage of total cancer burden contributable to CRC was 9.7% with 1.23 million cases, after lung (1.61 million) and breast cancer (1.38 million) [3]. In Europe, CRC was responsible for 212.000 (12.2%) deaths in 2008, representing the second most common cause of death by cancer after lung cancer (19.9%) [4;5]. Approximately 20-25% of patients with CRC already have metastatic disease at the time of diagnosis and 20-25% of patients will develop metastases during disease progression as well, resulting in a 40-45% high mortality rate [4;5].

Studies aiming at optimizing the diagnostic process and treatment of this disease are increasing, which probably caused CRC to be one of the most studied and best characterized processes of tumorigenesis. Through more biological knowledge of tumorigenesis in CRC, more emphasis on early detection and development of new and improved treatment regimens, mortality decreased with almost 5 percent over the last decade [2;6;7]. Unfortunately, overall mortality and morbidity rates in CRC still remain high [2].

Survival of CRC patients largely depends on the disease stage at diagnosis and varies widely between stages. Five-year survival for stage I is 93.6%, which drops drastically to 8.1% for stage IV patients [8]. Treatment of CRC comprises (radical) tumor resection and, depending on tumor stage, radio-or chemotherapy [9]. Treatment choices nowadays are influenced by the tumor, node and metastasis (TNM) classification of the Union for International Cancer Control (UICC) [10]. The TNM classification aims to provide an exact prediction system for prognosis, to guide therapy choices and to form an understandable and uniform 'cancer language' [11;12]. Over the past decades, this TNM staging has changed continuously. In 2009, the seventh edition of the TNM stage was published, replacing the sixth edition from 2002 [13]. Regrettably, the seventh TNM edition did not provide greater accuracy in predicting colorectal cancer patients' prognosis, but resulted in a more complex classification for daily clinical use [14].

Unfortunately, besides making tumor classification more complex over the past years, the TNM staging system was not able to provide the clinician with the optimal staging tool it was designed for. Furthermore, possible under-treatment or over-treatment of some patients groups might arise when using the TNM staging system for treatment allocation [10;15-17]. Studies have shown that approximately 20% to 25% of patients with lymph node-negative stage II colon cancer will suffer from recurrent disease within 5 years of follow-up [18;19]. These patients, also identified as high risk-stage II patients, might have benefited from adjuvant therapy, which they did not receive as this was not recom-

mended based on their defined TNM stage. Therefore, the use of TNM stage falls short in daily clinical practice, especially in identifying high-risk stage II patients, and needs to be supplemented with additional biomarkers that can improve the current staging and treatment allocation criteria substantially. The American Society of Clinical Oncology's Tumor Markers Expert Panel (ASCO TEMP-2006), The European Group on Tumor Markers (EGTM-2007) and The European Society of Medical Oncology (ESMO) have all reviewed the clinical applicability of widely studied biomarkers [20-23]. Interestingly, in spite of a tremendous amount of available literature on biomarkers in CRC, only a few biomarkers are used in daily clinical practice nowadays, like KRAS, BRAF, MSI and the Onco*type* DX Colon Cancer Assay (Table I). A possible explanation could be that most prognostic or predictive biomarkers are not validated in other (large) cohorts, or because there is lack of consensus in performing these studies, such as different antibodies used or different scoring methods, which makes their results incomparable [20]. Furthermore, the handling of tissues has been well recognized in contributing to assay variability and issues in assay validation [24]. Previously, a five step program for the introduction of biomarkers in clinical practice was developed with the first step being biomarker development in a preclinical, exploratory setting, subsequently followed by verification of this biomarker in a large retrospective study, validation and finally confirmation in a prospective randomized controlled trial [25].

In this review we aim to give an overview of the most studied biomarkers in CRC and we will emphasize on some difficulties and controversies studying these biomarkers. The main goal is to identify key biomarkers, which might have the potential to identify patients who can be spared from further treatment or for whom additional treatment is advised (prognostic biomarkers), and to identify which patients will benefit from therapy (predictive biomarkers), ultimately resulting in the use of precision medicine in the future.

Table I: Biomarkers used in clinical practice

Biomarkers	Clinical use
KRAS	Identification of resistance to anti-EGFR moAB in metastatic CRC patients
BRAF	Identification of resistance to anti-EGFR moAB in metastatic CRC patients Exclusion of Lynch Syndrome
MSI	Identification of Lynch Syndrome
Onco*type* DX Colon Cancer Assay	Inform treatment planning in stage II and II colorectal cancer patients

Abbreviations: MSI= Microsatellite Instability

PROGNOSTIC BIOMARKERS IN CRC

Microsatellite instability

Most cancers of the colon and rectum display a phenomenon termed genomic instability. There are two forms of genomic instability that reflect different genetic pathways of tumorigenesis. One form, called microsatellite instability (MSI), refers to a clonal change in the number of repeated DNA nucleotide units in microsatellites caused by deletions or insertions, and appears in tumors with deficient mismatch repair (MMR) [26]. The molecular phenotype of MSI was first described in CRC by an independent research group showing MSI as the hallmark of Lynch Syndrome, although it was not solely restricted to hereditary CRC [27]. The biochemical basis of this phenotype can be explained by strand-specific mismatch repair defects and was initially linked to germline mutations of the mismatch repair (MMR) gene *hMSH2* followed by the identification of mutations in another MMR gene, *hMLH1*. Only a short period here after, mutations in *PMS2* and *hMSH6* were found in Lynch Syndrome, completing the biological background of this MSI phenotype [27;28].

Currently, there are a few clinical criteria for MSI testing in CRC to select potential Lynch Syndrome patients to be candidates for molecular MSI testing (Bethesda Guidelines): 1: three or more relatives with CRC across ≥ 2 generations with one first-degree relative and one with a cancer age below 50 years; 2: CRC in a patient younger than 50 years of age; 3: synchronous or metachronous CRC regardless of age; 4: CRC with high-density tumor-infiltrating lymphocytes, Crohn's-like lymphocytic reaction, mucinous/signet-ring differentiation or medullary growth pattern, patient age ≤ 60 years; 5: CRC in ≥ one first-degree relative with CRC, with one cancer diagnosed in a patient with age ≤ 50 years; and 6: CRC in ≥ two first-/second-degree relatives with CRC at any age [29;30]. If there is a clinical suspicion of Lynch Syndrome, MSI testing with molecular screening and/or immunohistochemistry has been recommended by the ESMO Consensus Guidelines for management of patients with colon and rectal cancer and has been performed in clinical practice as well [23].

Contrary to Lynch Syndrome, a different mechanism causes the sporadic type of MSI to develop in CRC. This phenotype is associated with *hMLH1* promotor hypermethylation, resulting in lack of *hMLH1* expression and subsequently loss of mismatch repair system function [27]. If loss of *hMLH1* is observed by MSI testing, somatic hypermethylation of the *hMLH1* promotor should be considered. This sporadic type of MSI could be investigated through testing for a *BRAF V600E* mutation that is strongly associated with a sporadic origin or by analysis of *hMLH1* promotor hypermethylation [31].

It has been shown that MSI CRC is associated with a better prognosis than non-MSI CRC [32-36]. Therefore MSI might be introduced as a standard pathological assessment for patients not included in these guidelines as well. Unfortunately, results from studies

have been equivocal concerning the proposed survival benefit [37-41] and resulted in not routinely clinical testing for sporadic MSI CRC up till now. There are several reasons that prevent the introduction of MSI testing into standard pathological assessment. First, the prognostic effect of MSI is better appreciated for disease-specific survival than for overall survival [42]. This could be explained by the better prognosis of young patients (<50 years) with MSI CRC, as probably more young patients are likely to have Lynch Syndrome [33]. Including older patients with sporadic CRC, who can die of other diseases, might result in loss of positive prognostic effect of MSI in overall survival. Inclusion or exclusion of various age groups will likely influence the prognostic significance of MSI analysis. Nonetheless, several studies reported a favorable outcome for patients with MSI [37-41]. Second, the survival advantage of MSI might also be the result of less distant metastases at diagnosis, lower prevalence of advanced stage tumors, high prevalence of early stages at diagnosis and for the largest part by younger Lynch Syndrome cases [43]. In conclusion, MSI is a marker for better clinical outcome, but appears to be more pronounced for Lynch Syndrome [42].

The reluctance to introduce routine testing of MSI in clinical practice is also based on several other factors. First, clinicians may not be aware of the criteria and conditions requiring genetic screening and mutational analysis. Second, availability of a standardized laboratory test might not be sufficient. MSI testing in molecular pathology laboratories is becoming increasingly available, but requires expertise and experience in testing and interpretation. Nowadays, immunohistochemistry (IHC) shows high sensitivity and specificity in detecting MSI and could therefore offer a relatively cheap, easy to perform, and universally available test for MSI instead of a more complex polymerase chain reaction (PCR)-based MSI test [44]. Lastly, there are also socioeconomic issues to resolve, like ethical, legal and health care-related issues, before introducing MSI testing in clinical practice. Clearly, MSI has been used successfully in clinical practice for Lynch Syndrome diagnosis and also shows great clinical potential for routine testing of non-Lynch Syndrome CRCs, but first more research has to be performed on MSI in sporadic as well as hereditary CRC to truly understand the better clinical outcome of MSI.

KRAS

The RAS-family of oncogenes consists of three principal members, *KRAS, HRAS* and *NRAS*, which are all involved in tumor development [45]. *KRAS* is a proto-oncogene encoding a small 21 kD guanosine triphosphate/guanosine diphosphate binding protein modulating cellular proliferation and differentiation [46]. Active *KRAS* mutations are found in 35-42% of CRCs and are thought to occur early in CRC carcinogenesis. Almost 97% of all observed genetic events within *KRAS* are caused by seven different DNA base pair substitutions within codon 12 and 13 of exon 2, resulting in an amino acid substitution in the protein [47]. *KRAS* mutation was associated with a significantly higher risk of recur-

rence in the QUASAR study compared with wild-type *KRAS*, but not in the PETACC-3 study [23;47]. Other studies performed were also conflicting, with some finding a prognostic value of mutated *KRAS* alone, others finding this value concomitantly with mutated TP53 or *PIK3CA* and some reporting no prognostic value of mutated *KRAS* at all [47-51].

Differences in *KRAS* mutations at codon 12 and 13 may result in different biological and functional consequences that could influence the prognosis of CRC [52]. Initially, *KRAS* was found to be a strong prognostic factor in CRC, but this finding was later restricted to a codon 12 mutation, leading to a glycine to valine substitution (*G12V*) [53;54]. Therefore, larger studies are required to confirm whether a specific mutation is responsible for a clinically relevant prognostic effect.

An important reason for the discrepancies between the studies could be the study design of the individual studies. Data based on prospective analysis of a homogenous cohort treated and followed according to the highest clinical standards, as performed in a registration trial, are more robust and reliable than those arising from similar sized meta-analyses or retrospective studies. Therefore, well-performed clinical trials should be used to validate results on *KRAS* in order to resolve discrepancies.

In conclusion, the available data contradicts each other at this moment and does not support standard testing for *KRAS* mutations in clinical practice to identify patients with a worse prognosis, who might require more aggressive treatment. However, in a predictive setting, mutated *KRAS* has shown differentiation resistance to anti-EGFR monoclonal antibodies (135-139) and since then has been used in clinic for this purpose.

BRAF

The *BRAF* gene encodes a serine/threonine protein kinase belonging to the RAS-RAF-MEK-ERK kinase pathway regulated by *KRAS* protein activity and involved in CRC development [55;56]. Nearly all oncogenic transformations of *BRAF* are the *V600E* mutations [57]. A lot of studies investigated and confirmed the potential adverse prognostic impact of *BRAF* mutations [47;58-60]. Yokota *et al.* identified *BRAF* as an independent prognostic factor for survival in a retrospective cohort of 229 patients with advanced and recurrent CRC. Presence of this mutation was associated with a significantly higher risk of cancer–related death, independent of other confounding factors [60]. These findings were consistent with those of other recent studies using patients with both stage II and III disease and with studies including all stages [47;58;59;61].

The PETACC-3 and QUASAR studies showed no increased risk of relapse in stage II and III CRC patients, but PETACC-3 did show a worse overall survival (OS), particularly in patients with MSI-L or MSS tumors [34;47]. Two large retrospective studies are in accordance with these findings [57;59]. Samowitz *et al.* reported that the *BRAF V600E* mutation in MSS colon cancer was associated with a significantly poorer survival in stage II to IV colon cancer, but did not have an effect on the excellent prognosis of MSI tumors [57]. Some

patients in these trials were treated with cetuximab after relapse. Patients with mutated *BRAF* may not have benefitted from the survival advantage offered by this agent [62;63]. Therefore, the prognostic relevance of mutated *BRAF* on OS may have been overestimated. However, the outcome of patients with CRC having *BRAF* mutations is worse than that of patients with wild-type *BRAF* CRC, independent of treatment with cetuximab [64], which further strengthens *BRAF* as a marker for a worse chance of survival.

TP53

TP53 is a tumor suppressor gene on the short arm of chromosome 17 encoding a protein important in regulating cell division. P53 is normally expressed in case of DNA damage, resulting in growth arrest and apoptosis (programmed cell death) in rapidly dividing cells. In this way *TP53* functions as a tumor suppressor gene by aborting growth of potentially malignant cells [65]. Mutations of the *TP53* gene are detected in up to 85% of CRCs, usually occurring during the adenoma to adenocarcinoma transition[66]. Over the years *TP53* has been intensively studied as the genome guardian marker [67;68]. The immunohistochemical expression may have prognostic value in patients with CRC. Higher expression has been shown in tumors with lymph nodal involvement and 5-year survival is lower for patients with positive p53 staining [69]. In normal cellular conditions, synthesis and degradation of p53 are tightly regulated and the expression level is kept very low. In such conditions, p53 expression is generally not detectable by immunohistochemistry. Mutations in *TP53* lead to disruption of normal *TP53* function and accumulation of mutant p53 levels that are high enough to be detected by immunohistochemistry [70]. Lack of p53 staining with immunohistochemistry has been associated with wild-type *TP53*, indicating a functionally active *TP53*. On the contrary, high expression of p53 staining was associated with mutated *TP53* [71]. However, there is still debate on the use of mutational analysis or immunohistochemistry as a reliable marker for p53 dysfunction. Lack of consensus on antibodies and scoring might possibly be responsible for this [72;73]. Studies have shown that immunohistochemistry does not always match with mutation studies and that expression of mutant forms of p53 are not simply correlated to loss of *TP53 function* [70]. Cripps *et al.* reported that approximately 33% of the CRCs who do not show positive immunohistochemical staining of p53 do not have a detectable *TP53* mutation [74]. Also, a scattered positive immunohistochemical staining of p53 might represent a functionally active non-mutated *TP53* gene and must therefore be analyzed separately [73]. Most studies in the past, however, only analyzed positive staining versus negative staining [69;75-77]. Recently, Nyiraneza *et al.* investigated the value of immunohistochemistry of p53 in CRC [71]. In this study immunohistochemistry revealed 3 distinct staining patterns of p53 expression; complete negative staining associated with truncating *TP53* mutations, diffuse overexpression associated with missense *TP53* mutations and restricted overexpression associated with wild-type *TP53*. Furthermore, muta-

tion analysis by Lopez *et al*. showed that *TP53* mutations were only present in 79.6% of positively stained p53 tumors [70]. In 30.8% of the tumors with negative p53 staining *TP53* mutations were found as well, indicating no complete correlation between immunohistochemistry and mutation analysis based on RNA expression.

In summary, *TP53* could not be used as a prognostic marker so far. Lack of consensus on antibodies and scoring methods in immunohistochemical staining, lack of correlation between immunohistochemical overexpression and clinical data, and discrepancies between immunohistochemistry and mutation analysis, are responsible for contradicting results and are therefore important reasons for not justifying the use of *TP53* in clinical practice.

Apoptosis-related biomarkers

One of the most important hallmarks of cancer is their ability to evade programmed cell death or apoptosis [78]. During tumor development tumor cells can be triggered by lymphocytes of the patient's immune system, by accumulation of DNA damage, or by stress factors like growth factor deprivation, to undergo apoptosis [79;80]. The actual apoptotic cell death machinery, the part of the pathway responsible for the execution of apoptosis that results in the morphologic features characteristic for apoptosis, consists of a very complex cascade of interacting proteins. The key components are the caspases. Caspase-3 is activated at a point where the intrinsic and extrinsic apoptosis induction pathways converge. The level of activated caspase-3 should therefore give a reliable measure of ongoing apoptosis [81] and is widely used in studies for detection of apoptotic cells. Another marker often used and specific for apoptotic epithelial cells is M30, which recognizes a caspase-specific cleaved product of cytokeratin-18 [82].

Several publications have described the relevance of apoptosis for the clinical outcome in CRC patients with contradicting results [82-85]. Differences between these studies might have been caused by a different patient selection, a different method used and a different study design of these publications. There are also reasons to believe that differences exist as a result of microsatellite status of the tumor, location of the tumor in the bowel or biological differences between rectal and colon cancer [82-84;86]. Dolcetti *et al.* reported a high frequency of apoptosis in MSI tumors [86]. Jonges *et al*. described a higher expression of cleaved caspase-3 expression in right-sided tumors [84]. In some rectal cancer studies, low expression of apoptosis was related to more local recurrences [82;83]. In CRC, however, results were different with high expression of apoptosis related to more local recurrence [84]. Reasons for these discrepancies are unclear. As most rectal cancers are MSS, microsatellite status might possibly explain these differences. Location of the tumor might also have an important influence on apoptosis. Recently, the Cancer Genome Atlas Network attempted to find biological differences between colon and rectal cancer, but they only found differences in the anatomical tumor site

with more hypermethylation in right-sided tumors, which might be explained by the different embryonic origins of the right-and left-sided tumors [87]. Additional research on apoptosis, keeping the microsatellite status and the location of the tumor in mind, needs to be performed.

Furthermore, it might not be sufficient to study apoptosis on its own. A key factor in tissue homeostasis is the balance between the level of cell death and the level of proliferation. Two important hallmarks of tumorigenesis can cause disturbance of this balance; deregulation of the proliferative signaling pathway and deregulation of the apoptotic pathway [88;89]. Michael-Robinson *et al.* previously reported on a cohort of 100 colorectal cancer patients in which they determined an AI:PI ratio [90]. This Apoptotic Index: Proliferation Index was based on M30 IHC for the apoptosis level and Ki67 IHC for the proliferation index. They were able to relate their AI:PI index significantly to patient outcome. Preliminary data from our center also showed a better prognosis for patients with high levels of proliferation and apoptosis, especially in right-sided tumors (Reimers MS, Zeestraten ECM *et al.,* in progress). Therefore, further studies also need to be performed, which will focus on apoptosis as well as proliferation.

Ki67

Proliferation is one of the most important hallmarks tumor cells must acquire for tumorigenesis [78]. The proliferation activity of a tumor can be estimated by determining the expression levels of specific cell cycle-related antigens by using IHC. A widely used marker is the ki67 antigen, which is expressed in the nuclei during all cell cycle phases except during the G_0 phase [91]. High expression levels of ki67 have been shown to correlate with patient outcome in many types of cancers, such as breast cancer, malignant lymphomas and astrocytomas [92;93]. However, in colorectal cancer, there are discrepancies in the association of ki67 with prognosis and survival [94-96]. Most studies in CRC reported an inverse relationship between ki67 expression and patient outcome; thus patients with high expression of ki67 in their tumor sections showed a better chance of survival [76;90;94;96]. Still, discrepancies exist and the reasons for these remain unclear [94;96;97]. If we consider the balance between the level of cell death and the level of proliferation again as previously mentioned above, contradicting results between the different studies could be the result of differences in apoptosis in the tumor tissues, which were not evaluated in these ki67 studies simultaneously. High proliferation might be associated with survival advantages because these cells also undergo apoptosis resulting in tissue homeostasis. Michael-Robinson *et al*. showed that there was a significant correlation between the apoptotic index and proliferation index, indicating some degree of coordinated regulation [90]. However, a high ki-67 index was associated with improved survival in MSI tumors only and therefore microsatellite status might influence ki67 expression as well. Since most MSI tumors are found on the right side of the tumor, location of the

tumor might also influence ki67 expression [98]. Other studies on ki67 did not stratify for microsatellite status or location of the tumor [76;94;96]. In conclusion, contradicting results regarding ki67 expression exist. Further research should focus on combined analysis of proliferation and apoptosis, as a balance between these two hallmarks of cancer might exist. Furthermore, analyses should be stratified for microsatellite status and location of the tumor in order to truly understand the prognostic value of ki67.

Immune-related markers

Historically, the immune system has been attributed with an important role in controlling tumor growth and metastasis [99-101]. Evasion of immune surveillance and suppression of the immune system were therefore two important traits cancer cells had to acquire during the process of tumorigenesis [102]. Research from the last century has indicated that the effects the immune system has on tumor cells, both in the tumor microenvironment as well as during the process of tumor metastasis, can also contribute to tumor progression [103].

The first marker of tumor-immunogenicity is the level of HLA class I expression on cancer cells. Tumor cells can escape cytotoxic T-cell (CTL) recognition through downregulation or complete loss of HLA class I resulting in minimization of tumor-associated antigen (TAA) expression and subsequently less destruction of tumor cells by CTLs [100;101]. HLA class I expression has been shown to be of prognostic value in several types of solid cancer [104;105]. However, the results in CRC specifically have been contradicting [106-109]. Downregulation of HLA class I makes tumor cells more prone to Natural Killer (NK) cell destruction. Non-classical HLA-E and HLA-G also play an important role in immune surveillance by NK cells. Presence of HLA-E and HLA-G cause an inhibitory signal to NK cells, resulting in further immune escape [110-112]. Furthermore, immune reactivity can become suppressed by the attraction of immunosuppressive regulatory T cells (Tregs) into the tumor microenvironment [113;114]. The immunosuppressive effect of Tregs has been proven, with a high density of tumor-infiltrating Treg associated with an unfavorable prognosis in a wide range of human carcinomas, including breast and lung cancer [115;116]. However, in colon cancer different results are reported as well, with more Foxp3+ cells correlated with a better patient survival [117;118].

Microsatellite instability has been shown to be characterized by a specific immune response [119]. Accumulation of frameshift-derived-peptides (FSP) may contribute to immune recognition and dense lymphocyte infiltration observed in MSI tumors [119]. However, these tumors grow out to large tumor masses as well, possibly due to loss or downregulation of HLA class I, also frequently observed in these tumors [120]. Furthermore, MSI tumors showed a high infiltration of Tregs [119]. T cell responses in patients with MSI CRCs are frequently directed against selected microsatellite instability-induced FSP, possibly creating more immune-mediated tumor rejection [119;120]. Therefore, immune

escape mechanisms may play a role in tumors characterized by microsatellite instability, and thus both features should be considered when analyzing clinical prognosis in this tumor type.

Besides T cells, innate immune cells orchestrate an inflammatory environment that might inhibit or promote CRC development and progression as well, such as macrophages. Macrophages are a primary source of secreted pro-inflammatory cytokines, which are able to influence and stimulate growth and migration of tumor cells. For example, IL-6 released by macrophages directly promotes CRC cell progression. Furthermore, the interaction between IL-6 and IL-10 also influences CRC progression and prognosis by manipulating their microenvironment for tumor growth facilitation[121].

The interaction between tumor cells and immune cells is complex and multifaceted. As shown by our previous studies in breast cancer, immune markers are related to each other [104;122]. In our opinion, studies based solely on one immune marker are not sufficient. Therefore, more studies need to be performed which focus on combining immune markers. Also, contradicting results from previous studies need to be studied further, also taking into consideration microsatellite instability.

Genomic signatures

The recognition that molecular features of cancer, including gene expression profiles, are connected to clinical outcome has led to the development of molecular tests that provide important prognostic and predictive information to aid clinical decision making. Genomic Health Inc (Redwood City, CA) has developed four studies in stage II and stage III colon cancer, involving more than 1800 patients in total, where genomic profiling has identified genes that are predictive of recurrence in resected colon cancer patients who were treated with surgery alone or surgery + 5-FU/LV chemotherapy [123]. The results from these studies enabled the design of the 12-gene colon cancer Recurrence Score, which was then validated in a large, independent, prospectively designed study in stage II colon cancer patients from the QUASAR clinical trial. In the QUASAR validation study, the Oncotype DX® Colon Cancer Assay (the colon cancer Recurrence Score) was validated as a predictor of risk of recurrence in stage II colon cancer patients following surgery [124]. The Recurrence Score predicted recurrence risk independently of pathologic T stage, tumor grade, number of nodes examined, lymphovascular invasion, and microsatellite status, providing information not captured by the existing markers used in clinical practice. The Recurrence Score thus addressed individualized recurrence risk information needed for optimal treatment planning in stage II colon cancer. Since January 2010, the Onco*type* DX Colon Cancer Assay has been offered by the Genomic Health clinical laboratory under Clinical Laboratory Improvement Amendments (CLIA) standards for clinical use and is now available to support treatment planning for stage II and stage III colon cancer patients [125].

Furthermore, ColoPrint also showed promising results [126]. In this study a prognostic 18-gene signature was identified on the basis of unbiased gene selection, searching the whole genome for genes that had the highest correlation to a tumor relapse event. The signature was validated in an independent set of 206 patients with UICC stage I–III colon cancer from Barcelona, Spain, and in 135 clinical samples of patients with stage II colon cancer from Munich, Germany, using a diagnostic microarray platform.

Prior attempts have also been made to correlate gene expression profiles with recurrence in stage II and III colorectal cancer [127-130]. However, these studies have generally used fresh frozen tissues, which are less applicable in clinical practice, and have studied small patient cohorts and therefore lacked statistical power for convincing proof.

Genomic signatures potentially have a high prognostic value and some are already in use in clinical practice, like Onco*type* DX. Other genomic signatures need to be validated first before introducing them in clinical practice, preferably using tissues from randomized clinical trials.

PIK3CA

Activation of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway has been associated with the development of a number of human cancers, including CRC [131]. The *PIK3CA* gene encodes the p110 alpha catalytic subunit of PI3K [132]. Mutations in this gene have been identified in CRC, with most mutations localized in exon 9 and 20 [133]. Mutations have shown to activate the AKT-pathway, driving cell proliferation, and are present in 10-30% of all CRCs [134].

PIK3CA mutations were related to a worse chance of survival in CRC patients [135;136]. However, only a mutation in exon 20 might be responsible for this worse chance of survival [137]. Also, when stratified by *KRAS* status, a worse colon cancer-specific mortality associated with a *PIK3CA* mutation was only found in *KRAS* wild-type tumors [136].

18q Loss of Heterozygosity (LOH)

Allelic loss of 18q has been thought to occur late in the process of carcinogenesis and occurred in approximately 70% of CRCs. Deleted in Colon Cancer (DCC), SMAD4 and many other important candidate genes have been identified on 18q [134]. Patients who harbored a 18q LOH showed a worse OS [138;139], but other studies showed contradicting results [140;141]. Jen *et al*. showed that stage II and III patients with an intact 18q had a significantly better 5-years OS compared to patients with allelic loss of 18q, suggesting a prognostic role of 18q LOH [138]. A meta-analysis also showed that patients with 18q allelic imbalance and DCC loss of expression were associated with a worse survival compared to patients with an intact 18q and expression of DCC [134].

Unfortunately, some studies did not account for MSI status, which seemed to influence the association of 18qLOH with survival [47;142]. The prognostic effect of 18qLOH was

lost in multivariable analysis in these studies when accounted for MSI status. Therefore, the prognostic value of 18q LOH remains unclear. Validation is warranted to draw further conclusions.

CIMP

In the last few years, the existence of a new pathway for CRC pathogenesis has gained attention, which involves the transcriptional silencing of tumor suppressor genes by hypermethylation of CpG islands of the promoter region of various genes [143].

These tumors are classified as having the CpG island methylator phenotype (CIMP) [144]. One-third to one-half of all CRCs may evolve through this pathway [145]. CIMP tumors with methylation-induced silencing of *MLH1* constitute the majority of sporadic MSI CRCs [146].

However, most CIMP-positive tumors are associated with microsatellite stability (MSS).

These CIMP MSS tumors are comparable with MSI CRC on certain clinical and pathological features, including a predilection for females, advanced age of disease onset, predilection for proximal colon, poor differentiation and mucinous histology [147]. Jover *et al.* showed that CIMP did not influence disease free survival (DFS) and that patients with CIMP-positive tumors did not benefit from 5-FU-based adjuvant chemotherapy [147]. On the contrary, CIMP positive CRCs showed a worse overall survival after surgery alone. However, the same study reported that CIMP positive CRCs showed a better response to the combination of surgery and 5-FU treatment, which could be caused by aberrations in folate- or methyl group metabolisms in CIMP positive tumors [148]. Taken together, these studies might support that CIMP could be used as a prognostic marker, but further research is necessary to confirm and validate these data.

Chromosomal instability (CIN)

In addition to microsatellite instability and CIMP, the chromosomal instability (CIN) pathway is also involved in colorectal cancer pathogenesis. Most CRCs arise through this pathway, which is characterized by widespread imbalances in chromosome number (aneuploidy) and loss of heterozygosity [149]. CIN is observed in 65-70% of sporadic colorectal cancers. Defects in chromosomal segregation, telomere stability and the DNA damage response have been described, but the complete mechanism of CIN remains unclear [149].

The CIN phenotype was associated with a less favorable outcome for patients compared to tumors with MSI. Patients with CIN tumors showed a decreased overall and progression-free survival compared to patients with MSI tumors, irrespective of ethnic background, anatomic locations and adjuvant treatment with 5-FU [150]. In large meta-analyses the prognostic value of CIN has been established with a HR of 1.45 compared to CIN negative tumors [151].

In the future, the mechanisms that initiate CIN and the relationship between CIN and tumor progression need to be better defined in order to implement CIN as a biomarker in clinical practice.

PREDICTIVE BIOMARKERS IN CRC

The predictive markers in this review are divided in therapy-related predictive markers; chemotherapy- and aspirin-related; and predictive markers for treatment toxicities in CRC patients.

Therapy-related predictive biomarkers

Microsatellite instability

In addition to the positive prognostic influence of MSI in CRC, a predictive role for microsatellite status has been demonstrated by using data from randomized clinical trials of 5-FU-based therapy versus surgery-only control [152;153]. In these trials, treatment differed by MSI status and patients with MSI-high tumors who were treated with 5-FU-based therapy had a trend towards a worse outcome compared with surgery-alone controls. In contrast, other studies reported similar outcomes for MSI-high patients with chemotherapy [154] or even showed a greater benefit from 5-FU-treatment [36;155;156]. These contradictory results could be explained by the differences in study design, as these latest studies included patients who were not randomly assigned to 5-FU therapy versus control, thus allowing selection bias or other limitations inherent to nonrandomized studies. Also, Sinicrope *et al.* reported a positive reduction in disease progression rate in MSI CRC patients treated with 5-FU, but this was only due to the HNPCC cases [36]. Therefore, these cases need to be separated from the sporadic MSI cases in further studies.

Establishing microsatellite status could be of particular interest for stage II patients, where the modest therapeutic effect of 5-FU-based therapy (2-4% in 5-years DFS) emphasized the need for prognostic and predictive markers to risk-stratify these patients [157;158]. The favorable prognosis of MSI CRC patients and the lack of benefit from 5-FU based therapy in patients with MSI tumors support a non-adjuvant treatment approach. Therefore, if we could establish the predictive value of MSI in these patients, a lot of patients could be spared from over-treatment, expenses, treatment-related toxicities, and reduced quality of life during 5-FU-treatment.

Unfortunately, patients included in the previously mentioned studies were treated 20-30 years ago in multiple countries. The current standard for adjuvant therapy in CRC has changed over time. The current standard for stage III CRC nowadays is infusional fluorouracil, leucovorin and oxaliplatin. Preliminary data suggest that adding either

oxaliplatin or irinotecan to 5-FU/leucovorin may overcome possible MSI resistance to 5-FU treatment and thus even change the predictive value of MSI [159;160]. However, these recent data need further investigation and the available data so far do not justify excluding patients with stage III disease and MSI tumors from treatment according to current regiments.

KRAS

A randomized clinical trial conducted by the National Cancer Institute of Canada Clinical Trials Group (NCIG CTG) in collaboration with the Australasian Gastro-Intestinal Trials Group (AGITG) showed that among CRC patients who did not respond to advanced chemotherapy, monotherapy with cetuximab, a monoclonal antibody directed against the epidermal growth factor receptor (EGFR), improved their overall survival and progression-free survival and preserved their quality of life in comparison to best supportive care alone [161]. Cetuximab and panitumumab are registered for CRC patients whose tumors express EGFR protein as determined by immunohistochemistry. However, it has clearly been demonstrated that this method has no predictive value in terms of cetuximab activity in colorectal cancer, since there was no tendency towards a higher response rate with higher EGFR expression[162;163]. Furthermore, resistance to this treatment is common and might be explained by *KRAS*. *KRAS* can acquire activating mutations in exon 20 resulting in isolation of this pathway from the EGFR effect and thus rendering EGFR inhibitors, like cetuximab, ineffective [164-168]. Indeed, previous studies showed the ineffectiveness of cetuximab or other EGFR inhibitors for CRC patients bearing mutated *KRAS* [164-167]. Therefore, treatment of CRC patients with cetuximab, with all its costs and toxicities, would be most appropriate for CRC patients bearing wild-type *KRAS* only. Furthermore, the addition of EGFR-antibodies to chemotherapy for patients with *KRAS* mutations appeared to be detrimental [169]. *KRAS* mutation has thus emerged as the major negative predictor for EGFR therapy efficacy followed by clinical recommendation for use of patients with wild-type *KRAS* tumors only [170]. *KRAS* mutational testing of metastatic CRC has become a routine and is incorporated in many centers nowadays. However, not all patients with *KRAS* wild-type tumors benefit from cetuximab and panitumumab and the positive predictive value is low with a sensitivity of 47%. Additional markers are necessary to better identify which patients will benefit from EGFR therapy. Less frequently observed *KRAS* mutations beyond the well-studied codons 12 and 13, mutations in *NRAS*, *BRAF* and *PIK3CA* also showed that they are associated with resistance to anti-EGFR therapy [170].

BRAF

As written above, treatment decisions on cetuximab solely based on *KRAS*, with an occurrence of only 30-40% in nonresponsive patients [166;167;171;172], might not be adequate.

Therefore, the identification of additional markers of EGFR-targeted therapies in CRC is highly needed. Since EGFR triggers two main signaling pathways, the RAS-RAF-MAPK axis and the PI3K-PTEN-AKT pathway, resistance to anti-EGFR therapy could also be caused by other members of these pathways, like *BRAF* as part of the RAS-RAF-MAPK pathway [165]. *BRAF* is the principal downstream effector of *KRAS* [173;174]. Only a few studies on the relationship between *BRAF* and the effect of cetuximab were performed, both showing that BRAF mutations were related to resistance for EGFR-targeted therapies [62;63]. Although evidence is still inadequate to demonstrate a real association of *BRAF* mutations with non-responsiveness to anti-EGFR therapy, it has been recommended by the National Comprehensive Cancer Network (NCCN) for this purpose. Combined analysis of both *KRAS* and *BRAF* could be used to select patients eligible for EFGR-targeted treatment, with evident medical and economic implications. Further molecular markers are needed and more studies, especially a randomized controlled trial, need to be performed in order to confirm these results. Preliminary data suggest that the ineffectiveness of EGFR-targeted therapies could be restored by adding a *BRAF* inhibitor sorafenib concomitantly with cetuximab or panitumumab [63]. This treatment combination is currently undergoing clinical assessment in CRC in a trial sponsored by the National Cancer Institute (NCT00326495) and might be a promising discovery, but also requires further investigation. In addition to sorafenib, other compounds targeting either *BRAF* (PLX4032 and PLX4720) or its downstream effectors (ARRY-162, AZD6244, and PD0325901) are in clinical development and could be exploited in combination with EGFR-targeted therapy [175]. PLX4032 is a V600 BRAF inhibitor which showed promising results in melanoma. However, in CRC the clinical activity was modest, with only a 5% response rate. On the contrary, PLX4720 caused substantial delays in tumor growth, including tumor regression, without toxicities[176].

COX-2

Currently, the use of aspirin is gaining interest in CRC treatment. Aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to be effective in preventing colorectal cancer [177-179]. Aspirin inhibits cyclooxygenase-2 (COX-2), which is expressed in 70% of colorectal tumors and increases with a more-advanced stage of the disease [180;181]. COX-2 plays an important role in colorectal carcinogenesis, invasion, angiogenesis, and metastasis. Several studies have shown that selective COX-2 inhibitors are able to reverse this COX-2 effect [182]. Recent studies showed that aspirin might also play a role as adjuvant treatment in CRC [183;184]. Chan *et al.* showed that regular use of aspirin after a CRC diagnosis is associated with a lower risk of colorectal cancer–specific and overall mortality, especially for individuals with tumors that overexpress COX-2 [180]. Also, the same group reported that aspirin reduced the risk of CRC exclusively for individuals with elevated COX-2 expression [185]. Though these findings were from observational

studies, they confirmed experimental data that prostaglandins and non-prostaglandin COX-2 products are central to the pathogenesis of CRC. They are also in accordance with animal studies in which genetically modified mice had defective APC-genes and in which rats had CRC after administration of exogenous carcinogens [186]. Elevated COX-2 expression in genetic APC deficiency was related to enhanced tumorigenesis while deletion of the COX-2 gene had the opposite effect [187;188]. These data strongly suggest a central role of COX-2 in CRC and their inhibition as an effective chemopreventive measure. Unfortunately, studies investigating COX-2 expression for patients treated with aspirin are scarce, prompting the need for further validation of this possible biomarker.

Recent studies showed that aspirin not only influences COX-2 expression, but COX-1 inhibition might contribute to the antitumor effects of aspirin as well, for example at low-dose aspirin [189]. Experimental evidence also suggests additional COX independent actions of aspirin and other NSAIDs, like modifications of transcription factors (NFκB), induction of apoptosis and DNA stabilization [189].

Furthermore, aspirin use, even at low doses appropriate for cardiovascular risk management, is not without risks and roughly doubles the incidence of gastric bleeding [190]. These drugs have been shown to enhance cardiovascular risks as well [191]. Appropriate biomarkers are therefore needed to improve benefit/risk ratio. Since the exact mechanism of aspirin is not known yet, COX-2 tumor expression is not ready to be used as a biomarker to select CRC patients for aspirin treatment.

PIK3CA

In addition to the effect of COX-2 expression on aspirin treatment in CRC as written above, a recent study showed that only CRC patients bearing a mutation in PIK3CA (exon 9 or exon 20) benefitted from aspirin treatment and not patients with wild-type PIK3CA tumors [192].

The phosphatidylinositol 3-kinase (PI3K) signaling pathway plays an important role in carcinogenesis [133]. Mutations in *PIK3CA* are present in approximately 15 to 20% of colorectal cancers [48;193;194]. Up-regulation of PI3K enhances COX-2 activity and prostaglandin E2 synthesis, resulting in inhibition of apoptosis in colon-cancer cells [195]. Aspirin may suppress cancer-cell growth and induce apoptosis by blocking the PI3K pathway [196].

Unfortunately, only one study on the role of PIK3CA mutations in aspirin treatment in CRC has been performed so far, which had limited statistical power as well [192]. More studies are needed to validate these results and to unravel the therapeutic effect of aspirin in CRC.

Toxicity-related predictive biomarkers

DPD deficiency

Capecitabine, 5-fluorouracil (5-FU), and tegafur all belong to the fluoropyrimidines. Fluoropyrimidines are one of the most frequently used anti-cancer treatments in colorectal cancer with a good tolerability for most patients. However, in approximately 5-10% of the patients severe toxicity arises after treatment has started, which sometimes could be life threatening [197;198]. The intolerability of fluoropyrimidines is often caused by dihydropyrimidine dehydrogenase (DPD) deficiency, which is present in approximately 4% of the western population [199]. In 80% of patients with DPD deficiency the use of fluoropyrimidines in standard dose resulted in severe toxicity [200]. Screening for this intolerability could identify 'at risk' patients, resulting in less toxicity-related hospital admissions and lower medical costs. Also, treatment could be adjusted for these toxicities with lower doses or dose titration according to arising toxicities. Titration of the dose in DPD deficient patients could significantly reduce the frequency of severe, potentially deadly toxicity caused by fluoropyrimidines [201].

DPD deficiency can be determined by 'real-time' PCR, which is a simple technique and only requires 1 mL of blood with a sensitivity and specificity of 100% [202]. Unfortunately, current genotyping of DPD deficiency only detects 25-50% of all DPD deficient patients, as only DPYD*2A is detected so far, which has a frequency of 1-2% in the total population [203]. New mutations, which are related to fluoropyrimidine toxicity, have been identified, like DPYD 2846A>T and 1236G>A, and could be implemented in genotyping DPD deficiency [203;204]. In clinical practice, the lower toxicity associated with modern infusional or oral 5-FU based regimens make it impossible to screen the entire population for 30 polymorphisms associated with DPD deficiency. Despite the clear effect on toxicity, the prognostic and predictive value remains unclear with studies reporting contradicting results. Possibly, clinicians responded differently on the encountered toxicities. Despite well investigated evidence, the pharmocogenetic basis of varied DPD activity needs further investigation [151].

UGT1A1 Polymorphism

Irinotecan is a topoisomerase I inhibitor that interrupts DNA replication in cancer cells, resulting in cell death [205;206]. The irinotecan prodrug is activated by carboxylesterase to the active metabolite SN-38, which is 100–1000 times more cytotoxic than the parent drug [205]. SN-38 is further catalyzed into the inactive glucuronide derivative SN-38G by several hepatic and extrahepatic UGT enzymes. One of the major isozymes involved in this catalyzation is UGT1A1 [207]. A decrease in the level of functional UGT1A1 enzyme reduces a person's ability to metabolize SN-38 to an inactive form and is also associated with a higher risk of adverse side effects, like neutropenia and diarrhea caused by high

levels or prolonged exposure to the active form [208;209]. At least 63 *UGT1A1* variants have been described, including single base pair changes, frame shift mutations, insertions, and deletions in the promoter region, five exons and two introns of the gene. Most variants are associated with an absent, reduced, or inactive enzyme; one is associated with an increased enzyme level, and the effects of some variants are unknown [210]. Although, several clinical trials have confirmed that patients carrying different genotypes of *UGT1A1* had varied degrees of tolerance to irinotecan, it is still unclear whether *UGT1A1* has any influence on treatment efficacy. Three studies investigated the impact of *UGT1A1* isoforms on treatment outcome; however, their conclusions were inconsistent [211-213]. Many western studies have suggested that *UGT1A1*28* is significantly associated with irinotecan-induced toxicity [214-216]. In particular, patients bearing *UGT1A1*28 (TA7/7)* had a high possibility to develop severe neutropenia and diarrhea. Based on this, doctors are warned that patients with *UGT1A1*28 (TA7/7)* should start with a reduced dose of irinotecan, although the details on how to adjust the dose have not been specified [210]. On the other hand, research in Asian countries has shown a lower incidence of *UGT1A1*28 (TA7/7)*, while *UGT1A1*6 (A/A)* is more often found and may replace *UGT1A1*28* as a key regulator in UGT1A1 expression [217;218].

Palomaki *et al.* stated a few problems regarding the use of *UGT1A1* in clinical practice; there seems to be a clear relationship between *UGT1A1* genotype and severe neutropenia, but there is no direct or indirect evidence to support the clinical utility of modifying an initial and/or subsequent dose of irinotecan for patients with metastatic CRC as a way to change the rate of adverse drug events. Also, the data on the clinical validity of tests for *UGT1A1* variants other than **28* are limited and the analytic validity of *UGT1A1* testing in clinical practice is unknown. Laboratories offering such testing may include variants in addition to **28* for which little evidence is available. Furthermore, there are limited data on *UGT1A1* variants in Hispanic and African American populations. In order to recommend *UGT1A1* testing in clinical practice, additional studies are needed to understand the potential effects of alleles that are rare for Caucasians but more common for other racial/ethnic groups and studies should focus on all variants of clinical significance in the population [210].

DISCUSSION

Nowadays, knowledge about the process of tumorigenesis is increasing. As postulated by Hanahan *et al*. cancer cells must acquire biological capabilities during the multistep development of human tumors. Sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism and evading

immune destruction are all hallmarks of tumorigenesis [78]. Recognition of these concepts will increasingly affect the development of new treatment modalities in human cancer. Currently, recognition of these concepts has led to the identification of a lot of biomarkers, which might be of prognostic or predictive value in CRC.

Identifying and understanding molecular markers can improve the effectiveness of treatment in several ways; it may lead to the development of marker specific therapies and it may also improve the selection of adjuvant therapies by identifying those who will benefit most and therefore avoid toxic side effects for patients with the least risk of recurrence. The use of biomarkers might also have influence on social economical questions, decreasing the economic burden.

In this review we demonstrated the high potential of well-studied prognostic and predictive biomarkers in CRC. Only mutant *KRAS*, mutant *BRAF*, MSI and the Onco*type* DX Colon Cancer Assay are currently used in clinical practice for determining whether to treat metastatic CRC patients with cetuximab or panitumumab, for the evaluation of Lynch syndrome and to inform treatment planning in stage II and III colon cancer patients. Implementation of these biomarkers, however, has been beneficial. For example screening for MSI resulted in increased identification of patients with Lynch Syndrome [219].

Unfortunately, other biomarkers are not ready to be introduced in clinical practice, which can be explained by several factors. Firstly, study characteristics of the individual investigations on biomarkers varied widely. Sometimes a marker with prognostic significance was demonstrated, but only in a highly selected group of patients. Secondly, well-standardized protocols to detect the biomarker were not applied for any of the markers, particularly IHC. Also, there seemed to be no standardized method for quantification of the expression level of a certain biomarker. Lack of consensus in performing studies may greatly influence the interpretation of the results of these studies. If studies are not performed according to standardized protocols, it is extremely difficult to compare results of the individual studies. The handling of tissues has been well recognized as contributing to assay variability and issues in assay validation as well [24]. Some tissues are amenable to repeated sampling, without concern of substantial tissue heterogeneity or sampling issues, but often tissue-preserving methods cause damage or even destruction of tissues. New assays make great demands on the tissues , but it is impractical to replace the current tissue handling methods entirely. An integrated approach to the development and validation of integral biomarker assays might solve this problem. The difference between how a biospecimen is handled in a clinical setting and in a research setting must be reduced [24]. Thirdly, none of these biomarkers are validated in larger cohorts or even in prospective trials. Previously, a five step program for the introduction of biomarkers in clinical practice was developed with the first step being biomarker development in a preclinical, exploratory setting, subsequently followed by verification

of this biomarker in a large retrospective study, validation and finally confirmation in a prospective randomized controlled trial [25]. Unfortunately, this program has not been executed so far, which might explain why biomarkers are used so rarely in daily practice. Furthermore, most studies did not consider tumor heterogeneity, the influence of tumor - stromal interaction and the percentage of tumor in a sample, which also might influence results gained from molecular or immunohistochemical analyses. Finally, using only one marker to predict the outcome of patients seems inappropriate, as according to Hanahan *et al.* tumor cells acquire multiple capabilities for tumorigenesis [78]. Recently, our group has demonstrated that patients with both presence of HLA class I expression and Treg tumor infiltration had less relapses when treated with chemotherapy [220]. Combining markers might add more clinical value and gain more information about tumor aggressiveness.

In conclusion, the use of molecular markers and other biomarkers in CRC allows the identification of genes and biomarkers, which might predict individual prognosis and recurrence rate. Also, it might optimize treatment results and minimize treatment toxicities resulting in a decrease of economic burden and eventually the use of precision medicine in treating CRC patients. Only a few biomarkers are used in clinic nowadays. However, in order to introduce more biomarkers in clinical practice future studies need to consider the combination of markers, standardizing protocols and avoiding selection bias. Furthermore, simple, cheap, automated and standardized assays for the detection of molecular markers are necessary and most importantly, studies need to be validated in larger studies followed by prospective trials.

REFERENCE LIST

(1) Ferlay J, Parkin DM, Steliarova-Foucher E. Estimates of cancer incidence and mortality in Europe in 2008. *Eur J Cancer* 2010;46:765-781.

(2) Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011;61:69-90.

(3) Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010;127:2893-2917.

(4) La Vecchia C., Bosetti C, Lucchini F et al. Cancer mortality in Europe, 2000-2004, and an overview of trends since 1975. *Ann Oncol* 2010;21:1323-1360.

(5) Malvezzi M, Arfe A, Bertuccio P, Levi F, La VC, Negri E. European cancer mortality predictions for the year 2011. *Ann Oncol* 2011;22:947-956.

(6) Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. *CA Cancer J Clin* 2009;59: 225-249.

(7) Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin* 2010;60:277-300.

(8) O'Connell JB, Maggard MA, Ko CY. Colon cancer survival rates with the new American Joint Committee on Cancer sixth edition staging. *J Natl Cancer Inst* 2004;96:1420-1425.

(9) Cunningham D, Atkin W, Lenz HJ et al. Colorectal cancer. *Lancet* 2010;375:1030-1047.

(10) Greene FL, Sobin LH. The staging of cancer: a retrospective and prospective appraisal. *CA Cancer J Clin* 2008;58:180-190.

(11) Gospodarowicz MK, Miller D, Groome PA, Greene FL, Logan PA, Sobin LH. The process for continuous improvement of the TNM classification. *Cancer* 2004;100:1-5.

(12) Greene FL. TNM staging for malignancies of the digestive tract: 2003 changes and beyond. *Semin Surg Oncol* 2003;21:23-29.

(13) Sobin LH. *TNM classification of malignant tumours. 7th Ed. West Sussex, England, John Wiley & Sons*. John Wiley & Sons ed. 2009.

(14) Nitsche U, Maak M, Schuster T et al. Prediction of prognosis is not improved by the seventh and latest edition of the TNM classification for colorectal cancer in a single-center collective. *Ann Surg* 2011;254:793-800.

(15) Gunderson LL, Sargent DJ, Tepper JE et al. Impact of T and N stage and treatment on survival and relapse in adjuvant rectal cancer: a pooled analysis. *J Clin Oncol* 2004;22:1785-1796.

(16) Kahlenberg MS, Sullivan JM, Witmer DD, Petrelli NJ. Molecular prognostics in colorectal cancer. *Surg Oncol* 2003;12:173-186.

(17) Kozak KR, Moody JS. The impact of T and N stage on long-term survival of rectal cancer patients in the community. *J Surg Oncol* 2008;98:161-166.

(18) Chen SL, Bilchik AJ. More extensive nodal dissection improves survival for stages I to III of colon cancer: a population-based study. *Ann Surg* 2006;244:602-610.

(19) Davies M, Arumugam PJ, Shah VI et al. The clinical significance of lymph node micrometastasis in stage I and stage II colorectal cancer. *Clin Transl Oncol* 2008;10:175-179.

(20) Duffy MJ, van DA, Haglund C et al. Clinical utility of biochemical markers in colorectal cancer: European Group on Tumour Markers (EGTM) guidelines. *Eur J Cancer* 2003;39:718-727.

(21) Duffy MJ, van DA, Haglund C et al. Tumour markers in colorectal cancer: European Group on Tumour Markers (EGTM) guidelines for clinical use. *Eur J Cancer* 2007;43:1348-1360.

(22) Locker GY, Hamilton S, Harris J et al. ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. *J Clin Oncol* 2006;24:5313-5327.

(23) Schmoll HJ, Van CE, Stein A et al. ESMO Consensus Guidelines for management of patients with colon and rectal cancer. a personalized approach to clinical decision making. *Ann Oncol* 2012;23: 2479-2516.

(24) Hewitt SM, Badve SS, True LD. Impact of preanalytic factors on the design and application of integral biomarkers for directing patient therapy. *Clin Cancer Res* 2012;18:1524-1530.

(25) Pepe MS, Etzioni R, Feng Z et al. Phases of biomarker development for early detection of cancer. *J Natl Cancer Inst* 2001;93:1054-1061.

(26) Bolocan A, Ion D, Ciocan DN, Paduraru DN. Prognostic and predictive factors in colorectal cancer. *Chirurgia (Bucur)* 2012;107:555-563.

(27) Markowitz SD, Bertagnolli MM. Molecular origins of cancer: Molecular basis of colorectal cancer. *N Engl J Med* 2009;361:2449-2460.

(28) Laghi L, Bianchi P, Malesci A. Differences and evolution of the methods for the assessment of microsatellite instability. *Oncogene* 2008;27:6313-6321.

(29) Umar A, Boland CR, Terdiman JP et al. Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. *J Natl Cancer Inst* 2004;96:261-268.

(30) Vasen HF, Watson P, Mecklin JP, Lynch HT. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. *Gastroenterology* 1999;116:1453-1456.

(31) Capper D, Voigt A, Bozukova G et al. BRAF V600E-specific immunohistochemistry for the exclusion of Lynch syndrome in MSI-H colorectal cancer. *Int J Cancer* 2013.

(32) Bertagnolli MM, Redston M, Compton CC et al. Microsatellite instability and loss of heterozygosity at chromosomal location 18q: prospective evaluation of biomarkers for stages II and III colon cancer—a study of CALGB 9581 and 89803. *J Clin Oncol* 2011;29:3153-3162.

(33) Gryfe R, Kim H, Hsieh ET et al. Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer. *N Engl J Med* 2000;342:69-77.

(34) Hutchins G, Southward K, Handley K et al. Value of mismatch repair, KRAS, and BRAF mutations in predicting recurrence and benefits from chemotherapy in colorectal cancer. *J Clin Oncol* 2011;29: 1261-1270.

(35) Popat S, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. *J Clin Oncol* 2005;23:609-618.

(36) Sinicrope FA, Foster NR, Thibodeau SN et al. DNA mismatch repair status and colon cancer recurrence and survival in clinical trials of 5-fluorouracil-based adjuvant therapy. *J Natl Cancer Inst* 2011;103:863-875.

(37) Benatti P, Gafa R, Barana D et al. Microsatellite instability and colorectal cancer prognosis. *Clin Cancer Res* 2005;11:8332-8340.

(38) Chang EY, Dorsey PB, Johnson N et al. A prospective analysis of microsatellite instability as a molecular marker in colorectal cancer. *Am J Surg* 2006;191:646-651.

(39) Kim GP, Colangelo LH, Wieand HS et al. Prognostic and predictive roles of high-degree microsatellite instability in colon cancer: a National Cancer Institute-National Surgical Adjuvant Breast and Bowel Project Collaborative Study. *J Clin Oncol* 2007;25:767-772.

(40) Kohonen-Corish MR, Daniel JJ, Chan C et al. Low microsatellite instability is associated with poor prognosis in stage C colon cancer. *J Clin Oncol* 2005;23:2318-2324.

(41) Parc Y, Gueroult S, Mourra N et al. Prognostic significance of microsatellite instability determined by immunohistochemical staining of MSH2 and MLH1 in sporadic T3N0M0 colon cancer. *Gut* 2004;53:371-375.

(42) Guastadisegni C, Colafranceschi M, Ottini L, Dogliotti E. Microsatellite instability as a marker of prognosis and response to therapy: a meta-analysis of colorectal cancer survival data. *Eur J Cancer* 2010;46:2788-2798.

(43) Laghi L, Malesci A. Microsatellite instability and therapeutic consequences in colorectal cancer. *Dig Dis* 2012;30:304-309.

(44) Soreide K. Molecular testing for microsatellite instability and DNA mismatch repair defects in hereditary and sporadic colorectal cancers—ready for prime time? *Tumour Biol* 2007;28:290-300.

(45) Cipriano R, Graham J, Miskimen KL et al. FAM83B mediates. *J Clin Invest* 2012;122:3197-3210.

(46) Schubbert S, Shannon K, Bollag G. Hyperactive Ras in developmental disorders and cancer. *Nat Rev Cancer* 2007;7:295-308.

(47) Roth AD, Tejpar S, Delorenzi M et al. Prognostic role of KRAS and BRAF in stage II and III resected colon cancer: results of the translational study on the PETACC-3, EORTC 40993, SAKK 60-00 trial. *J Clin Oncol* 2010;28:466-474.

(48) Barault L, Veyrie N, Jooste V et al. Mutations in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in a population-based series of colon cancers. *Int J Cancer* 2008;122:2255-2259.

(49) Bazan V, Agnese V, Corsale S et al. Specific TP53 and/or Ki-ras mutations as independent predictors of clinical outcome in sporadic colorectal adenocarcinomas: results of a 5-year Gruppo Oncologico dell'Italia Meridionale (GOIM) prospective study. *Ann Oncol* 2005;16 Suppl 4:iv50-iv55.

(50) Conlin A, Smith G, Carey FA, Wolf CR, Steele RJ. The prognostic significance of K-ras, p53, and APC mutations in colorectal carcinoma. *Gut* 2005;54:1283-1286.

(51) Gonzalez-Aguilera JJ, Oliart S, Azcoita MM, Fernandez-Peralta AM. Simultaneous mutations in K-ras and TP53 are indicative of poor prognosis in sporadic colorectal cancer. *Am J Clin Oncol* 2004;27:39-45.

(52) Yokota T. Are KRAS/BRAF mutations potent prognostic and/or predictive biomarkers in colorectal cancers? *Anticancer Agents Med Chem* 2012;12:163-171.

(53) Andreyev HJ, Norman AR, Cunningham D, Oates JR, Clarke PA. Kirsten ras mutations in patients with colorectal cancer: the multicenter "RASCAL" study. *J Natl Cancer Inst* 1998;90:675-684.

(54) Andreyev HJ, Norman AR, Cunningham D et al. Kirsten ras mutations in patients with colorectal cancer: the 'RASCAL II' study. *Br J Cancer* 2001;85:692-696.

(55) Rajagopalan H, Bardelli A, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE. Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. *Nature* 2002;418:934.

(56) Davies H, Bignell GR, Cox C et al. Mutations of the BRAF gene in human cancer. *Nature* 2002;417: 949-954.

(57) Samowitz WS, Sweeney C, Herrick J et al. Poor survival associated with the BRAF V600E mutation in microsatellite-stable colon cancers. *Cancer Res* 2005;65:6063-6069.

(58) Farina-Sarasqueta A, van LG, Moerland E et al. The BRAF V600E mutation is an independent prognostic factor for survival in stage II and stage III colon cancer patients. *Ann Oncol* 2010;21: 2396-2402.

(59) Ogino S, Nosho K, Kirkner GJ et al. CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. *Gut* 2009;58:90-96.

(60) Yokota T, Ura T, Shibata N et al. BRAF mutation is a powerful prognostic factor in advanced and recurrent colorectal cancer. *Br J Cancer* 2011;104:856-862.

(61) Kakar S, Deng G, Sahai V et al. Clinicopathologic characteristics, CpG island methylator phenotype, and BRAF mutations in microsatellite-stable colorectal cancers without chromosomal instability. *Arch Pathol Lab Med* 2008;132:958-964.

(62) Benvenuti S, Sartore-Bianchi A, Di NF et al. Oncogenic activation of the RAS/RAF signaling pathway impairs the response of metastatic colorectal cancers to anti-epidermal growth factor receptor antibody therapies. *Cancer Res* 2007;67:2643-2648.

(63) Di Nicolantonio F., Martini M, Molinari F et al. Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *J Clin Oncol* 2008;26:5705-5712.

(64) Bokemeyer C, Van CE, Rougier P et al. Addition of cetuximab to chemotherapy as first-line treatment for KRAS wild-type metastatic colorectal cancer: pooled analysis of the CRYSTAL and OPUS randomised clinical trials. *Eur J Cancer* 2012;48:1466-1475.

(65) Levine AJ. p53, the cellular gatekeeper for growth and division. *Cell* 1997;88:323-331.

(66) Vogelstein B, Fearon ER, Hamilton SR et al. Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988;319:525-532.

(67) Lengauer C, Kinzler KW, Vogelstein B. Genetic instability in colorectal cancers. *Nature* 1997;386: 623-627.

(68) Leslie A, Pratt NR, Gillespie K et al. Mutations of APC, K-ras, and p53 are associated with specific chromosomal aberrations in colorectal adenocarcinomas. *Cancer Res* 2003;63:4656-4661.

(69) Menezes HL, Juca MJ, Gomes EG, Nunes BL, Costa HO, Matos D. Analysis of the immunohistochemical expressions of p53, bcl-2 and Ki-67 in colorectal adenocarcinoma and their correlations with the prognostic factors. *Arq Gastroenterol* 2010;47:141-147.

(70) Lopez I, Oliveira P, Tucci P, Alvarez-Valin F, Coudry A, Marin M. Different mutation profiles associated to P53 accumulation in colorectal cancer. *Gene* 2012;499:81-87.

(71) Nyiraneza C, Jouret-Mourin A, Kartheuser A et al. Distinctive patterns of p53 protein expression and microsatellite instability in human colorectal cancer. *Hum Pathol* 2011;42:1897-1910.

(72) Baas IO, Mulder JW, Offerhaus GJ, Vogelstein B, Hamilton SR. An evaluation of six antibodies for immunohistochemistry of mutant p53 gene product in archival colorectal neoplasms. *J Pathol* 1994;172:5-12.

(73) Kaserer K, Schmaus J, Bethge U et al. Staining patterns of p53 immunohistochemistry and their biological significance in colorectal cancer. *J Pathol* 2000;190:450-456.

(74) Cripps KJ, Purdie CA, Carder PJ et al. A study of stabilisation of p53 protein versus point mutation in colorectal carcinoma. *Oncogene* 1994;9:2739-2743.

(75) Buglioni S, D'Agnano I, Cosimelli M et al. Evaluation of multiple bio-pathological factors in colorectal adenocarcinomas: independent prognostic role of p53 and bcl-2. *Int J Cancer* 1999;84: 545-552.

(76) Garrity MM, Burgart LJ, Mahoney MR et al. Prognostic value of proliferation, apoptosis, defective DNA mismatch repair, and p53 overexpression in patients with resected Dukes' B2 or C colon cancer: a North Central Cancer Treatment Group Study. *J Clin Oncol* 2004;22:1572-1582.

(77) Kaklamanis L, Savage A, Whitehouse R et al. Bcl-2 protein expression: association with p53 and prognosis in colorectal cancer. *Br J Cancer* 1998;77:1864-1869.

(78) Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646-674.

(79) Collins MK, Perkins GR, Rodriguez-Tarduchy G, Nieto MA, Lopez-Rivas A. Growth factors as survival factors: regulation of apoptosis. *Bioessays* 1994;16:133-138.

(80) Hashimoto A, Hirose K, Iino M. BAD detects coincidence of G2/M phase and growth factor deprivation to regulate apoptosis. *J Biol Chem* 2005;280:26225-26232.

(81) Brown JM, Attardi LD. The role of apoptosis in cancer development and treatment response. *Nat Rev Cancer* 2005;5:231-237.

(82) de Bruin EC, van de Velde CJ, van de Pas S et al. Prognostic value of apoptosis in rectal cancer patients of the dutch total mesorectal excision trial: radiotherapy is redundant in intrinsically high-apoptotic tumors. *Clin Cancer Res* 2006;12:6432-6436.

(83) de Heer P., de Bruin EC, Klein-Kranenbarg E et al. Caspase-3 activity predicts local recurrence in rectal cancer. *Clin Cancer Res* 2007;13:5810-5815.

(84) Jonges LE, Nagelkerke JF, Ensink NG et al. Caspase-3 activity as a prognostic factor in colorectal carcinoma. *Lab Invest* 2001;81:681-688.

(85) Schwandner O, Schiedeck TH, Bruch HP, Duchrow M, Windhoevel U, Broll R. Apoptosis in rectal cancer: prognostic significance in comparison with clinical histopathologic, and immunohistochemical variables. *Dis Colon Rectum* 2000;43:1227-1236.

(86) Dolcetti R, Viel A, Doglioni C et al. High prevalence of activated intraepithelial cytotoxic T lymphocytes and increased neoplastic cell apoptosis in colorectal carcinomas with microsatellite instability. *Am J Pathol* 1999;154:1805-1813.

(87) Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 2012;487: 330-337.

(88) Bedi A, Pasricha PJ, Akhtar AJ et al. Inhibition of apoptosis during development of colorectal cancer. *Cancer Res* 1995;55:1811-1816.

(89) Takano Y, Saegusa M, Ikenaga M, Mitomi H, Okayasu I. Apoptosis of colon cancer: comparison with Ki-67 proliferative activity and expression of p53. *J Cancer Res Clin Oncol* 1996;122:166-170.

(90) Michael-Robinson JM, Reid LE, Purdie DM et al. Proliferation, apoptosis, and survival in high-level microsatellite instability sporadic colorectal cancer. *Clin Cancer Res* 2001;7:2347-2356.

(91) Scopa CD, Tsamandas AC, Zolota V, Kalofonos HP, Batistatou A, Vagianos C. Potential role of bcl-2 and ki-67 expression and apoptosis in colorectal carcinoma: a clinicopathologic study. *Dig Dis Sci* 2003;48:1990-1997.

(92) Bui MH, Visapaa H, Seligson D et al. Prognostic value of carbonic anhydrase IX and KI67 as predictors of survival for renal clear cell carcinoma. *J Urol* 2004;171:2461-2466.

(93) Kirla R, Salminen E, Huhtala S et al. Prognostic value of the expression of tumor suppressor genes p53, p21, p16 and prb, and Ki-67 labelling in high grade astrocytomas treated with radiotherapy. *J Neurooncol* 2000;46:71-80.

(94) Fluge O, Gravdal K, Carlsen E et al. Expression of EZH2 and Ki-67 in colorectal cancer and associations with treatment response and prognosis. *Br J Cancer* 2009;101:1282-1289.

(95) Kimura T, Tanaka S, Haruma K et al. Clinical significance of MUC1 and E-cadherin expression, cellular proliferation, and angiogenesis at the deepest invasive portion of colorectal cancer. *Int J Oncol* 2000;16:55-64.

(96) Salminen E, Palmu S, Vahlberg T, Roberts PJ, Soderstrom KO. Increased proliferation activity measured by immunoreactive Ki67 is associated with survival improvement in rectal/recto sigmoid cancer. *World J Gastroenterol* 2005;11:3245-3249.

(97) Pollack A, DeSilvio M, Khor LY et al. Ki-67 staining is a strong predictor of distant metastasis and mortality for men with prostate cancer treated with radiotherapy plus androgen deprivation: Radiation Therapy Oncology Group Trial 92-02. *J Clin Oncol* 2004;22:2133-2140.

(98) Jass JR, Do KA, Simms LA et al. Morphology of sporadic colorectal cancer with DNA replication errors. *Gut* 1998;42:673-679.

(99) Halvorsen TB, Seim E. Association between invasiveness, inflammatory reaction, desmoplasia and survival in colorectal cancer. *J Clin Pathol* 1989;42:162-166.

(100) Menon AG, Janssen-van Rhijn CM, Morreau H et al. Immune system and prognosis in colorectal cancer: a detailed immunohistochemical analysis. *Lab Invest* 2004;84:493-501.

(101) Murphy J, O'Sullivan GC, Lee G et al. The inflammatory response within Dukes' B colorectal cancers: implications for progression of micrometastases and patient survival. *Am J Gastroenterol* 2000;95:3607-3614.
(102) Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646-674.
(103) Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 2011;331:1565-1570.
(104) de Kruijf EM, van Nes JG, Sajet A et al. The predictive value of HLA class I tumor cell expression and presence of intratumoral Tregs for chemotherapy in patients with early breast cancer. *Clin Cancer Res* 2010;16:1272-1280.
(105) Madjd Z, Spendlove I, Pinder SE, Ellis IO, Durrant LG. Total loss of MHC class I is an independent indicator of good prognosis in breast cancer. *Int J Cancer* 2005;117:248-255.
(106) Benevolo M, Mottolese M, Piperno G et al. HLA-A, -B, -C expression in colon carcinoma mimics that of the normal colonic mucosa and is prognostically relevant. *Am J Surg Pathol* 2007;31:76-84.
(107) Menon AG, Morreau H, Tollenaar RA et al. Down-regulation of HLA-A expression correlates with a better prognosis in colorectal cancer patients. *Lab Invest* 2002;82:1725-1733.
(108) Moller P, Momburg F, Koretz K et al. Influence of major histocompatibility complex class I and II antigens on survival in colorectal carcinoma. *Cancer Res* 1991;51:729-736.
(109) Watson NF, Ramage JM, Madjd Z et al. Immunosurveillance is active in colorectal cancer as down-regulation but not complete loss of MHC class I expression correlates with a poor prognosis. *Int J Cancer* 2006;118:6-10.
(110) Cavallo F, De GC, Nanni P, Forni G, Lollini PL. 2011: the immune hallmarks of cancer. *Cancer Immunol Immunother* 2011;60:319-326.
(111) Hokland M, Kuppen PJ. Natural killer cells: from "disturbing" background to central players of immune responses. *Mol Immunol* 2005;42:381-383.
(112) Marin R, Ruiz-Cabello F, Pedrinaci S et al. Analysis of HLA-E expression in human tumors. *Immunogenetics* 2003;54:767-775.
(113) Nosho K, Baba Y, Tanaka N et al. Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer, and prognosis: cohort study and literature review. *J Pathol* 2010;222:350-366.
(114) Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat Rev Immunol* 2006;6:715-727.
(115) Bates GJ, Fox SB, Han C et al. Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol* 2006;24:5373-5380.
(116) Petersen RP, Campa MJ, Sperlazza J et al. Tumor infiltrating Foxp3+ regulatory T-cells are associated with recurrence in pathologic stage I NSCLC patients. *Cancer* 2006;107:2866-2872.
(117) Salama P, Phillips M, Grieu F et al. Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol* 2009;27:186-192.
(118) Suzuki H, Chikazawa N, Tasaka T et al. Intratumoral CD8(+) T/FOXP3 (+) cell ratio is a predictive marker for survival in patients with colorectal cancer. *Cancer Immunol Immunother* 2010;59:653-661.
(119) Bauer K, Nelius N, Reuschenbach M et al. T cell responses against microsatellite instability-induced frameshift peptides and influence of regulatory T cells in colorectal cancer. *Cancer Immunol Immunother* 2013;62:27-37.
(120) Bernal M, Garcia-Alcalde F, Concha A et al. Genome-wide differential genetic profiling characterizes colorectal cancers with genetic instability and specific routes to HLA class I loss and immune escape. *Cancer Immunol Immunother* 2012;61:803-816.

(121) Grizzi F, Bianchi P, Malesci A, Laghi L. Prognostic value of innate and adaptive immunity in colorectal cancer. *World J Gastroenterol* 2013;19:174-184.

(122) de Kruijf EM, Sajet A, van Nes JG et al. HLA-E and HLA-G expression in classical HLA class I-negative tumors is of prognostic value for clinical outcome of early breast cancer patients. *J Immunol* 2010; 185:7452-7459.

(123) O'Connell MJ, Lavery I, Yothers G et al. Relationship between tumor gene expression and recurrence in four independent studies of patients with stage II/III colon cancer treated with surgery alone or surgery plus adjuvant fluorouracil plus leucovorin. *J Clin Oncol* 2010;28:3937-3944.

(124) Gray RG, Quirke P, Handley K et al. Validation study of a quantitative multigene reverse transcriptase-polymerase chain reaction assay for assessment of recurrence risk in patients with stage II colon cancer. *J Clin Oncol* 2011;29:4611-4619.

(125) Kelley RK, Venook AP. Prognostic and predictive markers in stage II colon cancer: is there a role for gene expression profiling? *Clin Colorectal Cancer* 2011;10:73-80.

(126) Maak M, Simon I, Nitsche U et al. Independent Validation of a Prognostic Genomic Signature (ColoPrint) for Patients With Stage II Colon Cancer. *Ann Surg* 2013.

(127) Barrier A, Boelle PY, Roser F et al. Stage II colon cancer prognosis prediction by tumor gene expression profiling. *J Clin Oncol* 2006;24:4685-4691.

(128) Jiang Y, Casey G, Lavery IC et al. Development of a clinically feasible molecular assay to predict recurrence of stage II colon cancer. *J Mol Diagn* 2008;10:346-354.

(129) Lin YH, Friederichs J, Black MA et al. Multiple gene expression classifiers from different array platforms predict poor prognosis of colorectal cancer. *Clin Cancer Res* 2007;13:498-507.

(130) Salazar R, Roepman P, Capella G et al. Gene expression signature to improve prognosis prediction of stage II and III colorectal cancer. *J Clin Oncol* 2011;29:17-24.

(131) Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell* 2007;129:1261-1274.

(132) Engelman JA, Luo J, Cantley LC. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat Rev Genet* 2006;7:606-619.

(133) Samuels Y, Wang Z, Bardelli A et al. High frequency of mutations of the PIK3CA gene in human cancers. *Science* 2004;304:554.

(134) George B, Kopetz S. Predictive and prognostic markers in colorectal cancer. *Curr Oncol Rep* 2011; 13:206-215.

(135) He Y, Van't Veer LJ, Mikolajewska-Hanclich I et al. PIK3CA mutations predict local recurrences in rectal cancer patients. *Clin Cancer Res* 2009;15:6956-6962.

(136) Ogino S, Nosho K, Kirkner GJ et al. PIK3CA mutation is associated with poor prognosis among patients with curatively resected colon cancer. *J Clin Oncol* 2009;27:1477-1484.

(137) Farina SA, Zeestraten EC, van WT et al. PIK3CA kinase domain mutation identifies a subgroup of stage III colon cancer patients with poor prognosis. *Cell Oncol (Dordr)* 2011;34:523-531.

(138) Jen J, Kim H, Piantadosi S et al. Allelic loss of chromosome 18q and prognosis in colorectal cancer. *N Engl J Med* 1994;331:213-221.

(139) Watanabe T, Wu TT, Catalano PJ et al. Molecular predictors of survival after adjuvant chemotherapy for colon cancer. *N Engl J Med* 2001;344:1196-1206.

(140) Barratt PL, Seymour MT, Stenning SP et al. DNA markers predicting benefit from adjuvant fluorouracil in patients with colon cancer: a molecular study. *Lancet* 2002;360:1381-1391.

(141) Carethers JM, Hawn MT, Greenson JK, Hitchcock CL, Boland CR. Prognostic significance of allelic lost at chromosome 18q21 for stage II colorectal cancer. *Gastroenterology* 1998;114:1188-1195.

(142) Ogino S, Nosho K, Irahara N et al. Prognostic significance and molecular associations of 18q loss of heterozygosity: a cohort study of microsatellite stable colorectal cancers. *J Clin Oncol* 2009;27: 4591-4598.

(143) Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003;349:2042-2054.

(144) Hawkins N, Norrie M, Cheong K et al. CpG island methylation in sporadic colorectal cancers and its relationship to microsatellite instability. *Gastroenterology* 2002;122:1376-1387.

(145) Goel A, Arnold CN, Niedzwiecki D et al. Characterization of sporadic colon cancer by patterns of genomic instability. *Cancer Res* 2003;63:1608-1614.

(146) Kane MF, Loda M, Gaida GM et al. Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Res* 1997;57:808-811.

(147) Jover R, Nguyen TP, Perez-Carbonell L et al. 5-Fluorouracil adjuvant chemotherapy does not increase survival in patients with CpG island methylator phenotype colorectal cancer. *Gastroenterology* 2011;140:1174-1181.

(148) Van Rijnsoever M, Elsaleh H, Joseph D, McCaul K, Iacopetta B. CpG island methylator phenotype is an independent predictor of survival benefit from 5-fluorouracil in stage III colorectal cancer. *Clin Cancer Res* 2003;9:2898-2903.

(149) Pino MS, Chung DC. The chromosomal instability pathway in colon cancer. *Gastroenterology* 2010;138:2059-2072.

(150) Walther A, Houlston R, Tomlinson I. Association between chromosomal instability and prognosis in colorectal cancer: a meta-analysis. *Gut* 2008;57:941-950.

(151) Walther A, Johnstone E, Swanton C, Midgley R, Tomlinson I, Kerr D. Genetic prognostic and predictive markers in colorectal cancer. *Nat Rev Cancer* 2009;9:489-499.

(152) Ribic CM, Sargent DJ, Moore MJ et al. Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. *N Engl J Med* 2003;349: 247-257.

(153) Sargent DJ, Marsoni S, Monges G et al. Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. *J Clin Oncol* 2010;28:3219-3226.

(154) Kim GP, Colangelo LH, Wieand HS et al. Prognostic and predictive roles of high-degree microsatellite instability in colon cancer: a National Cancer Institute-National Surgical Adjuvant Breast and Bowel Project Collaborative Study. *J Clin Oncol* 2007;25:767-772.

(155) Elsaleh H, Joseph D, Grieu F, Zeps N, Spry N, Iacopetta B. Association of tumour site and sex with survival benefit from adjuvant chemotherapy in colorectal cancer. *Lancet* 2000;355:1745-1750.

(156) Hemminki A, Mecklin JP, Jarvinen H, Aaltonen LA, Joensuu H. Microsatellite instability is a favorable prognostic indicator in patients with colorectal cancer receiving chemotherapy. *Gastroenterology* 2000;119:921-928.

(157) Benson AB, III, Schrag D, Somerfield MR et al. American Society of Clinical Oncology recommendations on adjuvant chemotherapy for stage II colon cancer. *J Clin Oncol* 2004;22:3408-3419.

(158) Gill S, Loprinzi CL, Sargent DJ et al. Pooled analysis of fluorouracil-based adjuvant therapy for stage II and III colon cancer: who benefits and by how much? *J Clin Oncol* 2004;22:1797-1806.

(159) Bertagnolli MM, Niedzwiecki D, Compton CC et al. Microsatellite instability predicts improved response to adjuvant therapy with irinotecan, fluorouracil, and leucovorin in stage III colon cancer: Cancer and Leukemia Group B Protocol 89803. *J Clin Oncol* 2009;27:1814-1821.

(160) Zaanan A, Cuilliere-Dartigues P, Guilloux A et al. Impact of p53 expression and microsatellite instability on stage III colon cancer disease-free survival in patients treated by 5-fluorouracil and leucovorin with or without oxaliplatin. *Ann Oncol* 2010;21:772-780.

(161) Jonker DJ, O'Callaghan CJ, Karapetis CS et al. Cetuximab for the treatment of colorectal cancer. *N Engl J Med* 2007;357:2040-2048.

(162) Chung KY, Shia J, Kemeny NE et al. Cetuximab shows activity in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry. *J Clin Oncol* 2005;23:1803-1810.

(163) Saltz LB, Meropol NJ, Loehrer PJ, Sr., Needle MN, Kopit J, Mayer RJ. Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. *J Clin Oncol* 2004;22:1201-1208.

(164) Amado RG, Wolf M, Peeters M et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* 2008;26:1626-1634.

(165) Baselga J. The EGFR as a target for anticancer therapy—focus on cetuximab. *Eur J Cancer* 2001;37 Suppl 4:S16-S22.

(166) De Roock W., Piessevaux H, De SJ et al. KRAS wild-type state predicts survival and is associated to early radiological response in metastatic colorectal cancer treated with cetuximab. *Ann Oncol* 2008;19:508-515.

(167) Di Fiore F., Blanchard F, Charbonnier F et al. Clinical relevance of KRAS mutation detection in metastatic colorectal cancer treated by Cetuximab plus chemotherapy. *Br J Cancer* 2007;96:1166-1169.

(168) Fransen K, Klintenas M, Osterstrom A, Dimberg J, Monstein HJ, Soderkvist P. Mutation analysis of the BRAF, ARAF and RAF-1 genes in human colorectal adenocarcinomas. *Carcinogenesis* 2004;25: 527-533.

(169) Bokemeyer C, Bondarenko I, Makhson A et al. Fluorouracil, leucovorin, and oxaliplatin with and without cetuximab in the first-line treatment of metastatic colorectal cancer. *J Clin Oncol* 2009;27: 663-671.

(170) Vakiani E, Solit DB. KRAS and BRAF: drug targets and predictive biomarkers. *J Pathol* 2011;223: 219-229.

(171) Lievre A, Bachet JB, Le CD et al. KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res* 2006;66:3992-3995.

(172) Moroni M, Veronese S, Benvenuti S et al. Gene copy number for epidermal growth factor receptor (EGFR) and clinical response to antiEGFR treatment in colorectal cancer: a cohort study. *Lancet Oncol* 2005;6:279-286.

(173) Yan J, Roy S, Apolloni A, Lane A, Hancock JF. Ras isoforms vary in their ability to activate Raf-1 and phosphoinositide 3-kinase. *J Biol Chem* 1998;273:24052-24056.

(174) Zhang BH, Guan KL. Activation of B-Raf kinase requires phosphorylation of the conserved residues Thr598 and Ser601. *EMBO J* 2000;19:5429-5439.

(175) Friday BB, Adjei AA. Advances in targeting the Ras/Raf/MEK/Erk mitogen-activated protein kinase cascade with MEK inhibitors for cancer therapy. *Clin Cancer Res* 2008;14:342-346.

(176) De Roock W, De Vriendt V, Normanno N, Ciardiello F, Tejpar S. KRAS, BRAF, PIK3CA, and PTEN mutations: implications for targeted therapies in metastatic colorectal cancer. *Lancet Oncol* 2011;12: 594-603.

(177) Burn J, Gerdes AM, Macrae F et al. Long-term effect of aspirin on cancer risk in carriers of hereditary colorectal cancer: an analysis from the CAPP2 randomised controlled trial. *Lancet* 2011;378: 2081-2087.

(178) Rothwell PM, Fowkes FG, Belch JF, Ogawa H, Warlow CP, Meade TW. Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. *Lancet* 2011;377:31-41.

(179) Rothwell PM, Price JF, Fowkes FG et al. Short-term effects of daily aspirin on cancer incidence, mortality, and non-vascular death: analysis of the time course of risks and benefits in 51 randomised controlled trials. *Lancet* 2012;379:1602-1612.

(180) Chan AT, Ogino S, Fuchs CS. Aspirin use and survival after diagnosis of colorectal cancer. *JAMA* 2009;302:649-658.

(181) Midgley RS, McConkey CC, Johnstone EC et al. Phase III randomized trial assessing rofecoxib in the adjuvant setting of colorectal cancer: final results of the VICTOR trial. *J Clin Oncol* 2010;28: 4575-4580.

(182) Chen WS, Wei SJ, Liu JM, Hsiao M, Kou-Lin J, Yang WK. Tumor invasiveness and liver metastasis of colon cancer cells correlated with cyclooxygenase-2 (COX-2) expression and inhibited by a COX-2-selective inhibitor, etodolac. *Int J Cancer* 2001;91:894-899.

(183) Bastiaannet E, Sampieri K, Dekkers OM et al. Use of aspirin postdiagnosis improves survival for colon cancer patients. *Br J Cancer* 2012;106:1564-1570.

(184) Reimers MS, Bastiaannet E, van Herk-Sukel MP et al. Aspirin use after diagnosis improves survival in older adults with colon cancer: a retrospective cohort study. *J Am Geriatr Soc* 2012;60:2232-2236.

(185) Chan AT, Ogino S, Fuchs CS. Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. *N Engl J Med* 2007;356:2131-2142.

(186) Fischer SM, Hawk ET, Lubet RA. Coxibs and other nonsteroidal anti-inflammatory drugs in animal models of cancer chemoprevention. *Cancer Prev Res (Phila)* 2011;4:1728-1735.

(187) Oshima M, Dinchuk JE, Kargman SL et al. Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell* 1996;87:803-809.

(188) Williams CS, Smalley W, DuBois RN. Aspirin use and potential mechanisms for colorectal cancer prevention. *J Clin Invest* 1997;100:1325-1329.

(189) Schror K. Pharmacology and cellular/molecular mechanisms of action of aspirin and non-aspirin NSAIDs in colorectal cancer. *Best Pract Res Clin Gastroenterol* 2011;25:473-484.

(190) Elwood PC, Gallagher AM, Duthie GG, Mur LA, Morgan G. Aspirin, salicylates, and cancer. *Lancet* 2009;373:1301-1309.

(191) Garcia Rodriguez LA, Cea-Soriano L, Tacconelli S, Patrignani P. Coxibs: pharmacology, toxicity and efficacy in cancer clinical trials. *Recent Results Cancer Res* 2013;191:67-93.

(192) Liao X, Lochhead P, Nishihara R et al. Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. *N Engl J Med* 2012;367:1596-1606.

(193) Liao X, Morikawa T, Lochhead P et al. Prognostic role of PIK3CA mutation in colorectal cancer: cohort study and literature review. *Clin Cancer Res* 2012;18:2257-2268.

(194) Lievre A, Blons H, Laurent-Puig P. Oncogenic mutations as predictive factors in colorectal cancer. *Oncogene* 2010;29:3033-3043.

(195) Kaur J, Sanyal SN. PI3-kinase/Wnt association mediates COX-2/PGE(2) pathway to inhibit apoptosis in early stages of colon carcinogenesis: chemoprevention by diclofenac. *Tumour Biol* 2010;31: 623-631.

(196) Uddin S, Ahmed M, Hussain A et al. Cyclooxygenase-2 inhibition inhibits PI3K/AKT kinase activity in epithelial ovarian cancer. *Int J Cancer* 2010;126:382-394.

(197) de Gramont A., Figer A, Seymour M et al. Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. *J Clin Oncol* 2000;18:2938-2947.

(198) Hoff PM, Ansari R, Batist G et al. Comparison of oral capecitabine versus intravenous fluorouracil plus leucovorin as first-line treatment in 605 patients with metastatic colorectal cancer: results of a randomized phase III study. *J Clin Oncol* 2001;19:2282-2292.

(199) Mattison LK, Fourie J, Desmond RA, Modak A, Saif MW, Diasio RB. Increased prevalence of dihydropyrimidine dehydrogenase deficiency in African-Americans compared with Caucasians. *Clin Cancer Res* 2006;12:5491-5495.

(200) Deenen MJ, Cats A, Beijnen JH, Schellens JH. Part 4: pharmacogenetic variability in anticancer pharmacodynamic drug effects. *Oncologist* 2011;16:1006-1020.

(201) Deenen MJ, Cats A, Mandigers CM et al. [Prevention of severe toxicity from capecitabine, 5-fluorouracil and tegafur by screening for DPD-deficiency]. *Ned Tijdschr Geneeskd* 2012;156:A4934.

(202) Bosch TM, Bakker R, Schellens JH, Cats A, Smits PH, Beijnen JH. Rapid detection of the DPYD IVS14+1G>A mutation for screening patients to prevent fluorouracil-related toxicity. *Mol Diagn Ther* 2007;11:105-108.

(203) Deenen MJ, Tol J, Burylo AM et al. Relationship between single nucleotide polymorphisms and haplotypes in DPYD and toxicity and efficacy of capecitabine in advanced colorectal cancer. *Clin Cancer Res* 2011;17:3455-3468.

(204) van Kuilenburg AB, Meijer J, Mul AN et al. Intragenic deletions and a deep intronic mutation affecting pre-mRNA splicing in the dihydropyrimidine dehydrogenase gene as novel mechanisms causing 5-fluorouracil toxicity. *Hum Genet* 2010;128:529-538.

(205) Mathijssen RH, van Alphen RJ, Verweij J et al. Clinical pharmacokinetics and metabolism of irinotecan (CPT-11). *Clin Cancer Res* 2001;7:2182-2194.

(206) Miners JO, McKinnon RA, Mackenzie PI. Genetic polymorphisms of UDP-glucuronosyltransferases and their functional significance. *Toxicology* 2002;181-182:453-456.

(207) Nagar S, Blanchard RL. Pharmacogenetics of uridine diphosphoglucuronosyltransferase (UGT) 1A family members and its role in patient response to irinotecan. *Drug Metab Rev* 2006;38:393-409.

(208) Innocenti F, Undevia SD, Iyer L et al. Genetic variants in the UDP-glucuronosyltransferase 1A1 gene predict the risk of severe neutropenia of irinotecan. *J Clin Oncol* 2004;22:1382-1388.

(209) Van Cutsem E, Verslype C, Demedts I. The treatment of advanced colorectal cancer: where are we now and where do we go? *Best Pract Res Clin Gastroenterol* 2002;16:319-330.

(210) Palomaki GE, Bradley LA, Douglas MP, Kolor K, Dotson WD. Can UGT1A1 genotyping reduce morbidity and mortality in patients with metastatic colorectal cancer treated with irinotecan? An evidence-based review. *Genet Med* 2009;11:21-34.

(211) Cecchin E, Innocenti F, D'Andrea M et al. Predictive role of the UGT1A1, UGT1A7, and UGT1A9 genetic variants and their haplotypes on the outcome of metastatic colorectal cancer patients treated with fluorouracil, leucovorin, and irinotecan. *J Clin Oncol* 2009;27:2457-2465.

(212) Fujita K, Ando Y, Nagashima F et al. Genetic linkage of UGT1A7 and UGT1A9 polymorphisms to UGT1A1*6 is associated with reduced activity for SN-38 in Japanese patients with cancer. *Cancer Chemother Pharmacol* 2007;60:515-522.

(213) Toffoli G, Cecchin E, Corona G et al. The role of UGT1A1*28 polymorphism in the pharmacodynamics and pharmacokinetics of irinotecan in patients with metastatic colorectal cancer. *J Clin Oncol* 2006;24:3061-3068.

(214) Gagne JF, Montminy V, Belanger P, Journault K, Gaucher G, Guillemette C. Common human UGT1A polymorphisms and the altered metabolism of irinotecan active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38). *Mol Pharmacol* 2002;62:608-617.

(215) Shulman K, Cohen I, Barnett-Griness O et al. Clinical implications of UGT1A1*28 genotype testing in colorectal cancer patients. *Cancer* 2011;117:3156-3162.

(216) Strassburg CP, Kalthoff S, Ehmer U. Variability and function of family 1 uridine-5'-diphosphate glucuronosyltransferases (UGT1A). *Crit Rev Clin Lab Sci* 2008;45:485-530.

(217) Gusella M, Frigo AC, Bolzonella C et al. Predictors of survival and toxicity in patients on adjuvant therapy with 5-fluorouracil for colorectal cancer. *Br J Cancer* 2009;100:1549-1557.

(218) Minami H, Sai K, Saeki M et al. Irinotecan pharmacokinetics/pharmacodynamics and UGT1A genetic polymorphisms in Japanese: roles of UGT1A1*6 and *28. *Pharmacogenet Genomics* 2007; 17:497-504.

(219) Heald B, Plesec T, Liu X et al. Implementation of universal microsatellite instability and immunohistochemistry screening for diagnosing lynch syndrome in a large academic medical center. *J Clin Oncol* 2013;31:1336-1340.

(220) de Kruijf EM, van Nes JG, Sajet A et al. The predictive value of HLA class I tumor cell expression and presence of intratumoral Tregs for chemotherapy in patients with early breast cancer. *Clin Cancer Res* 2010;16:1272-1280.

CHAPTER 9

How does genome sequencing impact surgery?

Marlies S. Reimers*, Charla C. Engels*, Peter J. K. Kuppen, Cornelis J. H. van de Velde, Gerrit Jan Liefers

* Both authors contributed equally to this work

Nature Reviews Clinical Oncology, 2014 Oct;11(10):610-618

ABSTRACT

Cancer is a leading cause of death worldwide. Great efforts are dedicated to the development of prognostic and predictive biomarkers to improve diagnosis and achieve optimal treatment selection, thereby, introducing precision medicine in the multimodality treatment of cancer. Genomic aberrations are at the basis of tumor development, representing excellent candidates for the development of promising clinical biomarkers. Over the last decade, single-gene mutations and genomic profiling have been increasingly used in multidisciplinary consultations for risk-assessment and subsequent treatment planning for patients with cancer. We discuss the impact of such genetic-based information on surgical decision-making. Single-gene mutations have already influenced surgical decision-making in breast, colorectal and thyroid cancer. However, the direct impact of genomic profiling on surgical care has not yet been fully established. We discuss the direct and indirect influences of genomic profiling on surgery, and analyse the limitations and unresolved issues of a genotypic-approach to the surgical management of cancer.

INTRODUCTION

Despite early detection of cancer through screening programs and the development of new treatment modalities, the overall mortality as a consequence of this disease remains high [1]. The development of prognostic and predictive biomarkers for use in clinical practice has become a crucial part of cancer research. Single-gene mutations, which can be linked to cancer, have demonstrated promising prognostic and predictive value and have become increasingly used in multidisciplinary consultations for risk-assessment and subsequent individual treatment planning of patients with cancer [2-8]. Great examples are mutations within the *BRCA1* or *BRCA2* genes that are associated with a significantly increased risk of breast and ovarian cancer [9], and mutations in *KRAS,* which are extensively used for adjuvant treatment allocation in patients with colon cancer [2].

However, single-gene mutation analyses alone are unable to completely unravel the complexity of cancer. A more-global approach looking at changes in DNA, RNA or proteins that contribute to tumor growth and progression, is needed to capture the simultaneous interaction of many different mutated genes within malignant cells and their surrounding tissues. Genomic profiling, which enables gene expression profiles at a genome-wide level to be obtained, has already proven to have an impact on the diagnosis and prognostic classification of tumors, as well as on the prediction of response of individual patients to specific therapeutic regimens [10-12].

The promise of delivering precision medicine has been an incredibly strong driving force for the vast and rapid development of high-throughput genomic technologies. By definition, precision medicine is a multi-faceted approach to medicine that integrates molecular and clinical research with patient data and outcomes, with the aim of delivering a treatment targeted to the specific disease characteristics of an individual patient. Genomic, epigenomic, and environmental data are studied together with specific patient information to understand individual disease patterns and to design personalized preventive, diagnostic, and/or therapeutic solutions. Current regimens of cancer treatment are effective in a minority of patients, whereas adverse effects occur in many of the treated patients. Genome wide approaches may contribute to increase therapy benefit and decreasing adverse events by tailoring treatment decisions [13].

From a clinical perspective, the added value of genetic and genomic approaches is clear. However, their impact on surgery, which is still the cornerstone of cancer treatment, is less obvious. This Perspectives article discusses the effect and associated limitations of introducing single-gene mutations and genomic profiling in the surgical decision-making process in terms of timing, extent and subsequent treatment of the patient.

SINGLE-GENE MUTATIONS AND SURGERY

There are several examples of how single-gene mutations can guide surgical management, including mutations in *BRCA*1 and *BRCA2* in breast cancer, adenomatous polyposis coli (*APC*) in colorectal cancer (CRC), the mismatch repair genes (*MMR*) in hereditary colon cancer and other cancers, and *RET* in multiple encocrine-relared tumors [3;14-17].

BRCA mutations

Specifically, women carrying mutations in the tumor suppressor genes *BRCA1 or BRCA2* have a high (cumulative risk of 60–80%) lifetime risk of breast cancer [18]. The *BRCA* genes are normally expressed in breast cells and other tissues, where they have a crucial role in DNA damage repair. If a mutation occurs in one of these genes, DNA damage is not repaired properly, resulting in an increased risk of breast and ovarian cancer [19;20]. Nowadays, bilateral prophylactic mastectomy and oophorectomy are the most effective strategy available for risk reduction of breast and ovarian cancer in mutation carriers [15;20-22]. In a recent study, Neuburger *et al*. [23], showed that in the UK the number of women who had a bilateral mastectomy nearly doubled over the last decade, and more than tripled among women without breast cancer. Of note, bilateral prophylactic mastectomy has been shown to reduce breast cancer risk by 90% in *BRCA1* or *BRCA2* mutation carriers [24]. Despite this great risk reduction, nearly 64% of *BRCA1* or *BRCA2* carriers in the USA choose to avoid surgery as a result of the high sensitivity of MRI that allows early tumor detection [25]. Since ovarian cancer screening methods are largely ineffective, bilateral prophylactic salpingo-oophorectomy remains the standard of care in all *BRCA1* or *BRCA2* mutation carriers, leading to a risk reduction of 80-96% in women with *BRCA* associated gynaecologic cancers [26;27].

APC mutations

In CRC, familial adenomatous polyposis (FAP) is a syndrome in which the inherited defect in the gate-keeper tumor-suppressor *APC* gene leads to the development of multiple premalignant polyps throughout the colon as a result of uncontrolled growth, and subsequent malignant progression before the age of 40 years [28]. Therefore, a colectomy is advised after detection of a germ line mutation *APC*. Depending on the clinical features (such as patient age, the number, nature and location of polyps), a rectal or pouch-anal anastomosis is recommended [29]. Various aspects of surgical decision-making are influenced by both surgeons and patients, whose preferences should be taken into account with regard to optimal time for surgical intervention, extent of surgery and the type of anastomosis performed. Independent of mutation type, surgery will be recommended as soon as FAP syndrome is diagnosed because this is associated with an almost 100% risk of CRC [30]. However, since cancer is rare before the age of 20, surgery is often deferred

to the late teen years or in between major life changes, such as in academic transitions or between jobs [29]. The amount of polyps in the rectum are correlated with disease severity and are of crucial importance for deciding on the type of anastomosis [31]. When fewer than five rectal polyps are observed, an ileorectal anastomosis is advised as this correlated with mild disease. Conversely, if 20 or more rectal polyps are identified, indicating severe disease, an ileal pouch anal anastomosis will be recommended. Furthermore, morbidity quality of life and desired subsequent bowel function should be taken into account. Although pouch-anal anastomosis nearly eliminates CRC risk, it is associated with worse functional outcome, including an increased daily stool frequency, 24-hour incontinence, sexual dysfunction, decreased fecundity in females, impotence in men and decreased quality of life when compared to preservation of the rectum [32-35].

MMR mutations

Germline mutations in DNA *MMR* genes, *hMLH1*, *hMSH2*, *PMS2* or *hMSH6,* are responsible for another form of hereditary colon cancer, namely non-polyposis CRC (or Lynch Syndrome) [36]. MMR genes are involved in numerous cellular functions including DNA repair, apoptosis, anti-recombination and destabilization of DNA [37]. Lynch Syndrome is also associated with an increased risk of cancers of the stomach, small intestine, liver, bile ducts, upper urinary tract, brain, and skin [38;39]. Additionally, women with this disorder have a high risk of cancer of the ovaries and the endometrium [39]. Although the need for prophylactic surgery is less evident in Lynch syndrome patients than in FAP syndrome patients, those with Lynch syndrome who are diagnosed with CRC should consider total colectomy rather than a segmental colon resection due to the increased risk of metachronous neoplasia associated with this condition. A large observational study of 382 *MMR* gene mutation carriers (172 *MLH1*, 167 *MSH2*, 23 *MSH6* and 20 *PMS2*) followed for 9 years confirmed a high cumulative risk of metachronous CRC for 332 carriers treated by segmental resection for their primary CRC. In contrast, there were no diagnoses of metachronous CRC for the other 50 *MMR* gene mutation carriers treated by extensive colon resection [16].

RET mutations

Multiple endocrine neoplasia (MEN) are clinical inherited syndromes affecting different endocrine glands. The different patterns of MEN syndromes includes MEN1, MEN2A, MEN2B and medullary thyroid cancer (MTC) [17], which is commonly associated with pheochromocytoma (PHEO) and/or multiple adenomatosis of parathyroid glands with hyperparathyroidism (PHPT). These syndromes have very different clinical courses: MEN2B is very aggressive, MTC is almost indolent in most patients, and MEN2A is associated with variable degrees of aggressiveness [17]. Activating germline point mutations of the *RET* protooncogene—a 21-exon gene encoding for a tyrosine kinase transmem-

brane receptor involved in the transduction of signals for cell growth and differentiation—are present in 95% and 98% of families with MEN2A and MEN2B respectively, and in approximately 95% of families with MTC [17]. A presymptomatic gene diagnosis aimed at detecting the presence of *RET* mutations in patients with MEN2 syndrome has been established to improve morbidity and mortality for patients with this disease. The treatment of choice for primary MTC is total thyroidectomy with central neck lymph nodes dissection. However, even after radical surgery for MTC, there is a 30 percent chance of recurrence. Therefore, a prophylactic thyroidectomy is advised in patients with MEN2 carrying mutations in *RET* in order to guarantee a definitive cure and avoid morbidity of a central neck lymph node dissection [17].

The American Thyroid Association task force has suggested four different risk levels—from A (the lowest) to D (the highest)— for *RET* mutations , which are incorporated in their most recent management guidelines [40]. Specifically, children from families with MEN or MTC that carry *RET* mutations associated with a risk level D-(such as Met918Thr) should be surgically treated as soon as possible in the first year of life; whereas patients with level B and C risk levels (with *RET* mutations located in exons 10, 11, 13, 14, and 15) should be operated with a total thyroidectomy before 5 years of age; total thyroidectomy can be delayed till after the age of 5 or until the calcitonin positivity only for patients with a level A risk level (with *RET* mutations mapping to exon 5 and 8) [41]. Removing the thyroid in young children has a great impact on the child's life, as lifetime levothyroxine supplementation is required [42].

Recent data have shown that *RET* mutations carriers with undetectable levels of basal calcitonin have an almost no risk of developing MTC [43]. Moreover, serum levels of calcitonin <30–40 pg/ml are always associated with intrathyroidal micro-MTC without any evidence of lymph node metastases [43]. Elisei *et al.*[43] designed a study in which they operated on only *RET* mutation gene carriers depending on their basal and stimulated level of calcitonin. Total thyroidectomy was strongly indicated in patients when their basal or stimulated calcitonin levels were above 10 pg/mL. Importantly, this study showed that the time of surgical treatment could be personalized and safely planned once the positivity to calcitonin is detected at the annual assessment, independent of the type of *RET* mutation and its associated level of risk. This strategy obviously implies a high compliance of carriers of *RET* mutations to the scheduled follow-up if surgery is postponed as long as possible. The detection of mutations in the proto-oncogene *RET* has, therefore, become standard practice with surgical implications in MTC, that have crucially influenced the timing of surgery [41]. Furthermore, Xing *et al*. [44] have recently published an algorithm that incorporates cytology and molecular *(RET)* testing for the management of patients with thyroid nodules presenting with atypia of undetermined clinical significance, with the aim of limiting unnecessary and/or extensive surgery. This study suggests that in these patients, fine needle aspiration biopsy molecular analysis

should be performed for malignancy risk stratification. For example, a *BRAF* mutation in thyroid nodules from this specific patient group tends to be associated with increased risk of thyroid cancer and thus need for surgical intervention [44].

GENOMIC PROFILING

In the past decades, the technology for DNA and RNA analysis has evolved rapidly, shifting from single-gene mutation analysis to a genome wide, system-biology approach, well placed to assist in unravelling the complexity of cancer [5]. Since then, genomic profiling has been increasingly used in multidisciplinary consultations for risk-assessment and subsequent treatment planning for cancer patients. In the first part of this section the influence of these established RNA-based gene profiles on cancer management are discussed. The second part of this section focuses on the impact of genomic profiling on surgical decision-making in terms of timing and surgical extent.

Genome sequencing in cancer care

The first genome-wide approaches used to predict clinical outcome in patients with cancer were based on RNA microarray analyses [45]. In one study that used microarray analysis, a panel of 50 genes identified low-risk and high-risk lung cancer patients with significantly different survival outcomes. Since then, many RNA expression profiles have been published with varying clinical value (Table 1).

Specifically, the Onco*type* DX® profile (Genomic Health Inc., Redwood City, CA) showed a promising prognostic value and also proved beneficial for adjuvant treatment allocation for patients with breast cancer [46]. In this assay, the recurrence score is calculated using a 21-gene assay, which includes 16 cancer-related genes and five reference genes for standardization, and determined a recurrence risk estimate (low, intermediate, or high) for each patient [46]. In breast cancer, the recurrence score proved to be an independent predictor of distant recurrence in patients with node-negative, estrogen receptor (ER)-positive breast cancer treated with tamoxifen. The recurrence score was also shown to be a predictor of the magnitude of chemotherapy benefit, with patients with high recurrence score showing the greatest benefit from chemotherapy [46;47]. The recurrence score was also found to be prognostic and predictive for postmenopausal patients with hormone receptor-positive disease and with positive nodes who were treated with tamoxifen. However, these studies showed no benefit from chemotherapy in patients with low recurrence scores [10;47].

These results were validated in a separate study, in which the prognostic value of the recurrence score for postmenopausal hormone receptor-positive, node-negative and –positive patients with breast cancer treated with aromatase inhibitors was also

Table 1: Established RNA based prognostic and predictive profiles for breast and colorectal cancer

Breast Cancer Profiles				
Test	**Company**	**Technique**	**Proven value**	**Tissue requirements**
Onco*type* DX	Genomic Health, Inc. (Redwood City CA, USA)	qRT-PCR (21 genes)	Prognostic	Fresh frozen or FFPE
MammaPrint	Agendia BV (Amsterdam, Netherlands)	Micro-array based gene expression profiling (70 genes)	Prognostic	RNA of fresh tissue cores or frozen material or FFPE
Colorectal Cancer Profiles				
Onco*type* DX	Genomic Health Inc. (Redwood City CA, USA)	qRT-PCR (12 genes)	Prognostic	Fresh frozen or FFPE
ColoPrint	Agendia BV (Amsterdam, the Netherlands)	Micro-array based gene expression profiling (18 genes)	Prognostic	Fresh frozen material

Abbreviations; CI, confidence interval; CT, chemotherapy; ER+, oestrogen receptor positive; ET, endocrine therapy; FFPE, formaline fixed paraffin embedded; HR, hazard ratio; LN-, lymph node negative; LN+, lymph node positive; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; RS, recurrence score.

Output	Results	Validation	References
RS: *Low: <18* *Intermediate: 18-31* *High: ≥31* 10-years distant recurrence risk for ER+ve, LN- BC patients	*Low risk:* 6.8% chance of distant recurrence (95%CI 4.0-9.6) *Intermediate risk:* 14.3% (95%CI 8.3-20.3) *High risk:* 30.5% (95% CI 23.6-37.4) *High risk* & LN-: significant benefit from CT (HR 0.26 (95%CI 0.13-0.53)). Same is seen for LN+ (HR 0.59). Not seen in *low risk* patients	Yes Current Prospective trials: TAILORx: LN- patients RxPONDER: LN+ patients	46, 47
Mammaprint risk score: *Low & high risk* to develop metastasis in five years follow-up in BC patients	*Low vs. High*: HR 4.6 (95%CI 2.3-9.2) Sensitivity>90%	Yes Current prospective trial: MINDACT: LN-/LN+ patients	12, 50, 51
RS: *Low: <18* *Intermediate: 18-31* *High: ≥31* 10-years distant recurrence risk in stage II colon cancer patients	Chance of distant recurrence in 3 years: -*Low risk:* 12% -*Intermediate:* 18% -*High risk:* 22% High vs. Low risk: HR 1.47 (95% CI 1.01-2.14)	Yes	11, 54
Coloprint risk score: *low & high risk* to develop metastasis in five years follow-up for stage II and III colon cancer patients	Five years distant metastasis free survival: -*Low:* 94.9% -*High:* 80.6% High vs. Low risk: HR 4.28 (95%CI 1.36-13.5)	Yes	55, 56

demonstrated [48]. Furthermore, recent findings have also suggested that the recurrence score is able to predict locoregional recurrence (LRR) in patients with node-negative ER-positive breast cancer treated with tamoxifen [49]. This same study further showed that patients who underwent a mastectomy had significantly less LRR compared with patients who received lumpectomy followed by breast radiotherapy. When subdivided by age categories (<50 or ≥50 years), patients aged <50 years with high recurrence score seemed to have better clinical benefit from mastectomy than from lumpectomy and radiotherapy. On the basis of these results, patients with breast cancer, aged <50 years, featuring a high recurrence score should be advised to undergo a mastectomy.

In addition to the Onco*type* DX® profile, the MammaPrint® (Agendia Inc. , Amsterdam, The Netherlands) RNA mini-array was developed for use in the high-throughput clinical setting for the diagnosis of breast cancer [12;50;51]. Using a supervised classification method, the correlation coefficient of the expression for approximately 5,000 genes was correlated with disease outcome in a retrospective cohort of 78 patients [12]. Classification was made on the basis of the correlations of the expression profile of the 'leave-one-out'sample with the mean expression levels of the remaining samples from the good and the poor prognosis patients, respectively. The accuracy improved until the optimal number of marker genes was reached (70 genes). In a validation study, this prognostic profile was tested in 295 consecutive patients. The estimated HR for distant metastases in the group with a poor-prognosis signature, was 5.1 (95% CI, 2.9-9.0; $p<0.001$) [51]. MammaPrint® is a 70-gene prognosis profile that was reported to be superior to standard clinical parameters, such as nodal status and grade, in predicting the occurrence of distant metastasis in patients with breast cancer [51]. Moreover, the MammaPrint® profile also showed predictive value in patients assigned to the 'high-risk' subgroup, who had a significant benefit of 12% for combined (chemotherapy and hormone therapy) treatment when compared with patients in the low risk subgroup [52]. Once available, the results of the randomized controlled trial MINDACT (Microarray in Node-negative Disease may Avoid ChemoTherapy) will contribute to the validation of the predictive role of MammaPrint® [53].

As in breast cancer, one of the clinically established RNA profiles for colon cancer is the Onco*type* DX® profile. This profile was established from four studies performed in over 1,800 patients with stage II or stage III colon cancer [54]. Genomic profiling in these studies allowed the identification of seven genes associated with tumor recurrence risk, six genes associated with chemotherapy benefit and five reference genes, that were predictive of recurrence in patients with resected colon cancer who were treated with surgery alone or surgery followed by 5-Fluorouracil and Leucovorin chemotherapy. This analysis led to the design of a 12-gene colon cancer recurrence score, which was validated in the QUASAR clinical trial [11]. According to this 12-gene score, predefined risk groups are categorized as low, intermediate or high risk for tumor recurrence, which

gives the possibility to allocate high-risk stage II colon cancer patients to adjuvant treatment, ultimately protecting patients from costly overtreatment. Of note, currently the Onco*type* DX® assay has prognostic value regarding outcome in colon cancer, however, no predictive value has been established for adjuvant treatment so far.

In addition, the ColoPrint® (Agendia,Amsterdam, The Netherlands), a prognostic 18-gene signature that was identified through unsupervised hierarchical clustering of a whole-genome oligonucleotide high-density microarray leading to unbiased gene selection, also showed promising results in patients with colon cancer [55]. The signature was validated in an independent set of patients with stage II colon cancer and identified a 5-year distant metastasis-free survival of 94.9 ± 2.2% for low-risk patients and 80.6 ± 6.6% for high-risk patients, (p=0.009) [56]. These results support the prognostic value of RNA profiling in patients with stage II colon cancer and herewith facilitate the identification of patients who may benefit from chemotherapy. Nevertheless, surgical treatment will not change at all, using this type of prognostication.

High-throughput genomic analysis have led to the identification of different genomic signatures (or profiles) that can be used for cancer management and can contribute to the multidisciplenary decision making process for cancer treatment. However, as described in the following section, the direct impact of genomic profiling on surgery, timing and/or extent of the procedure, is currently less clear.

Impact of genomic profiles on surgery

Breast cancer

Several studies have shown that gene expression profiling of biopsies is a succesful tool that can predict response to neo-adjuvant treatment [57;58]. Specifically, Ayers *et al.*[57] suggested that transcriptional profiling had the potential to identify a 74-gene expression pattern on biopsies of breast cancer that might lead to clinically useful predictors of pathological complete response (pCR) to the neo-adjuvant treatment regimen of sequential weekly paclitaxel in combination with 5-fluorouracil, doxorubicin and cyclophosphamide. However, this small sample study still needs further validation. Chang *et al.*[58] analysed core biopsy samples from 24 patients with breast cancer and found an association of a 92-gene signature with treatment response to neo-adjuvant monotherapy with docetaxel. These studies suggest that genomic-profiling on biopsies represents a clinically relevant progress in cancer management. It can be argued that current practice should focus on genomic profiling of the tumor biopsy, before assignment of a targeted neo-adjuvant treatment. Although this aspect does not have a direct impact on surgery, it could influence the extent and timing of surgery indirectly (Figure 1). Targeted neo-adjuvant treatment could potentially lead to downsizing of the tumor, with consequently less-extensive surgery or even a delay in surgery in case of

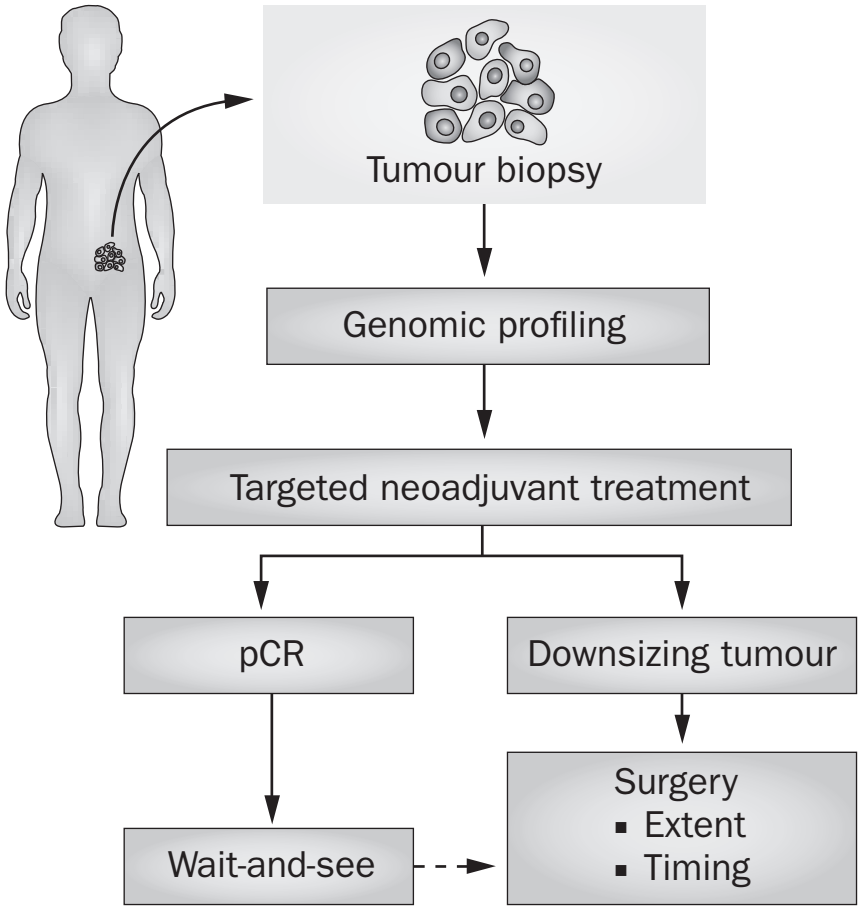


Figure 1: Impact of genomic profiling on surgery.
This figure shows two ways that genomic profiling might impact surgical intervention. Through genomic profiling of a tumor biopsy targeted neo-adjuvant treatment can be administered to a patient, possibly resulting in pathological complete response (pCR) or downsizing of the tumor. Downsizing of the tumor might influence surgery with regards to extent or timing of surgery. In instances of pCR a wait-and-see approach can be followed, where surgery is no longer necessary and a strict follow-up is advised.

a clinical complete response (cCR). By using genomic profiling to tailor neo-adjuvant treatment, response rates may increase. This will result in lower mastectomy rates.

In breast cancer, there is already a shift from mastectomy to breast-conserving surgery after tumor shrinkage by neo-adjuvant chemotherapy, which proved to be oncologically safe in terms of survival outcomes [59;60]. This decrease of mastectomy rates is a result of response to chemotherapy. Although this response can be predicted by molecular profiling of the tumor, the surgical planning in itself is not directly influenced by any gene expression signature. For local control, the studies by Cho *et al.*[59] and Shin *et al*. [60], investigating the oncologic safety of conservative surgery versus mastectomy after neo-adjuvant chemotherapy also improved outcome in terms of local recurrence. However, the number of patients included and the number of local events were too small to draw a significant conclusion in terms of therapeutic safety. These studies imply that through

targeted neo-adjuvant treatment, based on biopsy profiling, further downsizing of the tumor could occur and result in less invasive surgery. Today there are no known genomic profiles that guide surgical planning directly for breast cancer. Perhaps in the future, the risk of local regional recurrences can be predicted on the basis of genomic profiling in such a way that even after excellent response to neo-adjuvant therapy, a mastectomy is advised.

Pancreatic cancer

An other example of the potential impact of genomic profiling of biopsies is pancreatic cancer. Neo-adjuvant chemotherapy with gemcitabine and docetaxel in patients with borderline resectable cancer of the pancreatic head showed that operative exploration was associated with curative intent in 48% of the patients investigated [61]. Of the patients that underwent surgery, 87% had a R0 resection and 10% had a complete pathological response. This treatment was associated with a low perioperative morbidity and favourable survival: 81% of patients with resected cancers were alive at a median follow-up of 21.6 months [61]. Although this result was not directly based on genomic profiling, it is expected that genomic analysis of these tumors (both mutation analysis and expression profiling) will better identify 'treatment sensitive'tumor characteristics, which may lead to optimization of allocation of directed neoadjuvant treatment per individual patient.

In the future, a more curative surgical intervention could be achieved for patient groups with limited resection options, as a result of genomic profiling of the tumor biopsy, when therapeutic regimens are further optimized by targeted neo-adjuvant treatments.

Rectal cancer

As described above, neo-adjuvant treatment sometimes leads to downstaging of the primary tumor or even a complete clinical or pathological response. Therefore, more R0 resections and less-extensive surgeries can be achieved. With the use of genomic profiling on biopsy samples, followed by targeted neo-adjuvant treatement, the impact on surgical intervention can be striking, possibly leading to the omission of surgery. One can argue that based on specific genomic profiles from tumor biopsies, a wait-and-see approach might be indicated following complete clinical response after tailored neo-adjuvant therapy [62]. With this wait-and-see approach surgery can be delayed or even omitted. In patients with rectal cancer, this wait-and-see approach, however, is under debate. Curative total mesorectal excision after preoperative chemoradiation is the current standard of care in rectal cancer, in which pCR is observed in nearly 14% of these patients [63]. This example highlighted the rationale of a wait-and-see policy, which was further suggested by the results from a series of retrospective studies from Brazil. The Brazilian studies reported similar survival rates in patients that after complete clinical

response following neo-adjuvant treatment underwent radical resection or observation only [64-68]. Furthermore, Maas *et al.* [69] showed that a wait-and-see policy with strict selection criteria, up-to-date imaging techniques and follow-up is feasible with promising rates of 89% and 100% for cumulative probabilities of 2-year disease-free survival and overall survival, respectively, in patients with rectal cancer showing a complete clinical response. However, this study was small with a low local event rate, making clinical significance debatable. Recently, a study investigating criteria for determination of residual disease after neo-adjuvant chemotherapy showed that the majority of patients with a complete clinical response still had pathological residual disease [70]. For maximal benefit from a wait-and-see approach in rectal cancer, we should aim for better identification of patients with pathological complete response.

Oesophageal cancer

In oesophageal cancer, neo-adjuvant treatment can downstage tumors, thereby increasing R0 resections [71]. In one study, patients were randomly assigned to surgery alone or to chemoradiotherapy with carboplatin and paclitaxel followed by surgery [71]. Complete resection with no tumor within 1 mm of the resection margins (R0) was achieved in 92% of patients in the chemoradiotherapy-surgery group versus 69% in the surgery group ($p<0.001$). A pCR was achieved in 47 of 161 patients (29%) who underwent resection after chemoradiotherapy. In this scenario, targeted neo-adjuvant therapy based on the genomic profile of a biopsy was shown to influence surgery by improving the R0 resections and pCR rates.

In patients with locally advanced oesophageal cancer, the benefit from neo-adjuvant chemoradiotion is clear, but the benefit from surgery afterwards is less obvious [72]. Some patients with oesophageal cancer will have a pCR after neo-adjuvant chemoradiation and some of these patients would be able to forego surgery, but unfortunately evidence to guide treatment is scarce. For patients with squamous cell oesophageal cancer, those with a good clinical response after neo-adjuvant chemoradiation do not have a worse survival when undergoing observation only compared to surgery after chemoradiation [73]. The absolute benefit from surgery after neo-adjuvant chemoradiation seems to be relatively modest for patients with a good clinical response [72]. In selected patients with a complete clinical response following neo-adjuvant treatment, 3-year survival rates of 50% are seen irrespective of subsequent surgical intervention [74]. The accurate prediction of response to neo-adjuvant therapy can, therefore, have a direct influence on the surgical management of cancer. As treatment regimens improve and detection of earlier-stage disease increases (resulting in higher percentages of pCR), alternative approaches for patients at high risk of morbidity from surgery should be sought [75]. Even though evidence is not derived from randomized controlled trials, it might be reasonable to forego surgical intervention in patients with a complete clinical response,

especially in elderly with comorbidities who are less fit to undergo surgery and more likely to experience adverse events. On the basis of these results, one can imagine that genomic-profiling could have an additional role in targeting the tumor with the most optimal neo-adjuvant treatment, possibly leading to an even better local control and survival outcome. However, in current clinical practice, this approach has not been routinely established yet.

FUTURE DEVELOPMENTS

Genomic profiling is gaining importance in the multidisciplinary treatment of cancer. A direct impact on surgical oncology, however, cannot yet be claimed. Genomic testing on biopsies could potentially affect surgical management, but some important issues still remain unresolved and warrant further investigation before genomic profiling on biopsies can truly influence surgical decision-making.

First, several studies in different types of cancer have shown that in most cases sufficient tissue can be obtained from biopsies for performing genomic profiling [76;77]. However, in 20% of the cases limited tissue quantity is available from a biopsy, precluding further analysis [76]. Furthermore, low tumor content may need more in-depth sequencing or even a repeated biopsy to obtain more material for analysis, which is undesirable from the patient perspective. Therefore, improvement of profiling techniques is necessary to allow the identification of a valid profile in these more complicated circumstances.

Second, the risk of tumor seeding while performing the biopsy should not be underestimated. Case reports of malignant seeding following needle-biopsy have in fact been described in several tumors [78-80]. However, the clinical significance of this seeding is not known. In breast cancer, although data are limited, no increased morbidity has been observed as a consequence of tumor seeding [81].

Third, the heterogeneous nature of the tumor could contribute to unreliable prognostication and prediction. Genomic and epigenomic factors, among others, contribute to this heterogeneity and, consequently, newly developed targeted anti-cancer drugs will only be effective in a subset of patients, and perhaps only at a specific stage of their disease. A biopsy represents only a small fraction of the primary tumor, and owing to the heterogeneity of the tumor, important information could be missed, possibly resulting in a misleading phenotype. A solution for this issue is to obtain multiple biopsy samples from several locations throughout the tumor, although a higher risk of tumor seeding may be a consequence of this increased sampling.

Finally, the interactions of the tumor with the micro-environment influence tumor development and maintenance [82]. These patient-specific factors challenge adequate tumor sampling for biomarker discovery, warranting the use of techniques such as laser capture

microdissection for separate analysis of tumor and normal tissue for biomarker profiling. Some profiles, such as MammaPrint®, were derived from the analysis of tissue sections containing both the tumor and its closely surrounding micro-environment, whereas others, such as Onco*type* DX® , analysed only cancer cells. The different gene signatures identified from these approaches reveal a great variety of differentially expressed genes, with minimal overlap between the signatures identified. For example, Varga *et al.* [83] showed that nearly 18% of breast cancer patients showed major-discrepancy between Endopredict and Onco*type* DX® assay. In current clinical practice, the use of these techniques would require highly trained personnel and are associated with high costs and, therefore, is not advisable. It is important to implement sample handling, processing and data analysis into a routine standardized practice, thereby increasing quality of the array and decreasing costs and inter-laboratory variability [84].

Lack of clarity regarding how to assess a pCR, the ideal timing for a clinical, radiological and pathological assessment of response, the uncertainty of the long-term efficacy of this strategy and new follow-up protocols are all factors that currently influence the surgical implication of genomic profiling [85]. Of note, the decision of when to have surgery after chemoradiation is still an important issue. Patients should be given adequate time to recover from chemoradiation-associated toxic effects and sufficient time should be allowed for the tumor to respond to treatment. The optimal time-frame between neo-adjuvant treatment and surgery remains unclear and is most probably dependent on the specific tumor as well as on the individual patient. However, retrospective data in patients with rectal cancer and oesophageal cancer indicate that, in general, delaying surgery after neo-adjuvant therapy improves neo-adjuvant treatment response and decreases surgical complications [86;87]. These studies reported an increased pCR rate among patients who had a greater time frame between neo-adjuvant treatment and surgery [86;87], and an improved 5-year survival and a lower recurrence rate [88].

Finally, an important issue is that if genomic profiling is performed on tumor biopsies prior to the targeted neo-adjuvant treatment, the genomic signature identified might not be factual as the treatment could alter the genomic profile of the remaining tumor, possibly resulting in unreliable prognostication and prediction of adjuvant treatment benefit owing to this prespecified genomic profile [62]. Hannemann *et al.*[62] analyzed changes in gene expression patterns of breast tumors induced by chemotherapy, and compared the profiles of the pretreatment tumor-biopsy with the profiles of the remaining tumors after treatment. The researchers found that major changes in gene expression in locally advanced breast cancer were observed in responders to neo-adjuvant treatment, defined as patients with a tumor shrinkage >50%, but not in patients with resistant tumors [62]. Furthermore, Buchholz *et al.*[89] showed that genomic profiles of biopsies obtained from one patient before treatment or 24h and 48h after initiation of treatment clustered together more than samples obtained from different patients with

comparable tumor stage [89]. The fact that no differences were observed before and after treatment in the study from Buchholz *et al.*[89] might be due to the time-points chosen for the biopsies. In fact, changes in gene expression might only occur at later time points (after 48 h). From a surgeon's perspective, neo-adjuvant-induced tumor shrinkage is desirable as it leads to less extensive surgery with a higher chance of free surgical margins. However, not knowing the blueprint of the tumor left behind when radical surgery is avoided still leaves us in the dark. Overall, the value of this prespecified genomic tumor biopsy profile before neo-adjuvant treatment is largely unknown, owing to the fact that redetermination of the genomic profile of the remaining tumor after neo-adjuvant treatment cannot be ruled out.

CONCLUSION

The multimodality treatment of cancer has witnessed an increasing influence of genomic profiling in clinical decision-making. The complex interplay of genetic and epigenetic alterations in our genomes leads to disrupted biochemical interactions in multiple pathways, which are responsible for tumor development (Box 1). Ultimately, identifying these genomic abnormalities will lead to accurate prediction of tumor recurrence or to cancer-related death, non-responsiveness to therapy, and might even provide potential new targets for cancer therapy.

Box 1: Impact of epigenetic changes on surgery

Epigenetics, including DNA methylation and histone modifications, is defined as the study of inherited changes in gene expression or cellular phenotype, caused by mechanisms other than changes in the underlying DNA sequence. Epigenetic changes have shown to be critical for the development and progression of all cancer types [93-95]. Of note, these changes are intrinsically reversible and are therefore attractive targets for therapeutic intervention [93;96-98]. Drugs for both DNA methyl transferases (DNMTs) and histone deacetylases (HDACs), involved in addition of methylgroups to DNA and removal of acetyl groups on histone tails, are available [99;100]. DNMT inhibitors have shown promising results in cancer therapy, but unfortunately their activity is genome-wide rather than targeting specific genes [101]. A number of HDAC inhibitors have been designed to drive re-expression of aberrantly silenced genes, leading to inhibition of cell proliferation, hormone receptor reactivation and/or apoptosis [102]. In the future, these directed epigenetic treatments could potentially have the same impact on surgery as seen with targeted

neo-adjuvant chemotherapy after biopsy profiling. Furthermore, epigenetic changes can be detected in tumor-derived DNA in stool, tissues or blood [103-105], allowing the use of epigenetic markers in a clinical setting. This advance could lead to earlier tumor detection with an indirect impact on surgical care, influencing extent and timing of surgery with less delay in surgical intervention [106].

In prostate cancer, DNA hypermethylation of glutathione *S*-transferase pi 1 (*GSTP1*) [107] can be detected in urine, serum and ejaculate [108], which was able to increase sensitivity of prostate cancer diagnosis [109] and distinguish between primary cancer tissue and benign tissue [110].

In CRC, identification of hypermethylation of *P16* [111] , *DAPK* (death associated protein kinase)[112], *RUNX3* [113] and *ALX4* (aristaless like homeobox-4) [114] in blood or stool also served as a screening tool. Recently, a panel of highly sensitive and specific biomarkers for methylated DNA in plasma was identified, which resulted in three genes (*TMEFF2*, *NGR2* and *SEPT9*) specific in discriminating healthy subjects from patients with colorectal neoplasia [115].

It is hoped that these screening methods will lead to earlier tumor detection, however, this will not necessarily translate to increased survival and reduced mortality. Future studies, especially randomized controlled trials are warranted to tackle these issues and increase sensitivity of this exciting diagnostic field.

In current clinical practice, surgery still is the cornerstone of cancer treatment and the most valuable outcome predictor. Whereas some single-gene mutations described here have successfully impacted on cancer surgery, genomic tumor profiling has no direct impact on surgical decision-making, thus far. Today's research, however, is showing promising results, in particular genomic profiling of tumor biopsies, before and/or after targeted neo-adjuvant treatment, may result in less-extensive surgical techniques owing to optimal tumor shrinkage, or even lead to a wait-and-see approach.

The data disscussed in this Perspectives article are mainly derived from retrospective analyses in prospectively designed studies. These studies were not conducted in a randomized setting; therefore, confounding may be present. Furthermore, patient numbers were often limited, thereby decreasing statitiscal power and clinical significance. Currently, two large randomized controlled trials in the adjuvant setting are ongoing, where according to risk stratification using Onco*type* DX® or MammaPrint®, patients are randomly assigned for adjuvant chemotherapy in the TailorX or Mindact Trial, respectively [53;90]. The results of these trials will help define the true surgical implication of genomic profiling.

More comparable trials, for example, in the neo-adjuvant setting, are needed with the aim of limiting the extent of surgery.

Molecular targeted therapy might radically alter cancer treatment in the future and have the potential to greatly improve cancer survival by delivering the most effective drugs to the right patients [91]. Nevertheless, the treatment of cancer, especially in older patients or in patients with multiple comorbidities, should also take into account these comorbid conditions, quality of life, patient resilience, and preferences. Despite the great contribution of genetics and genome profile to cancer therapy, considering only the sum of genetic aberrations in cancer is insufficient for developing and deciding adequate cancer treatment, especially in elderly patients. In the USA, the estimated number of cancer patients older then 65 years of age will rise from 850,000 cases in 2012 to 1.3 million in 2025 [92]. This population is characterized by a great heterogeneity in terms of comorbidities, quality of life and patient preferences. These factors are as crucial as the molecular signature of the tumor in the multidisciplinary approach to cancer. Thus, phenotypic profiling must be part of the vanguard of cancer research (Figure 2).

In conclusion, genomic profile-directed cancer therapy is still in its infancy. Much more is expected from this field of research, which might contribute to precision medicine in the future of cancer treatment. Currently, it is not clear if genomic profiling will ever gain

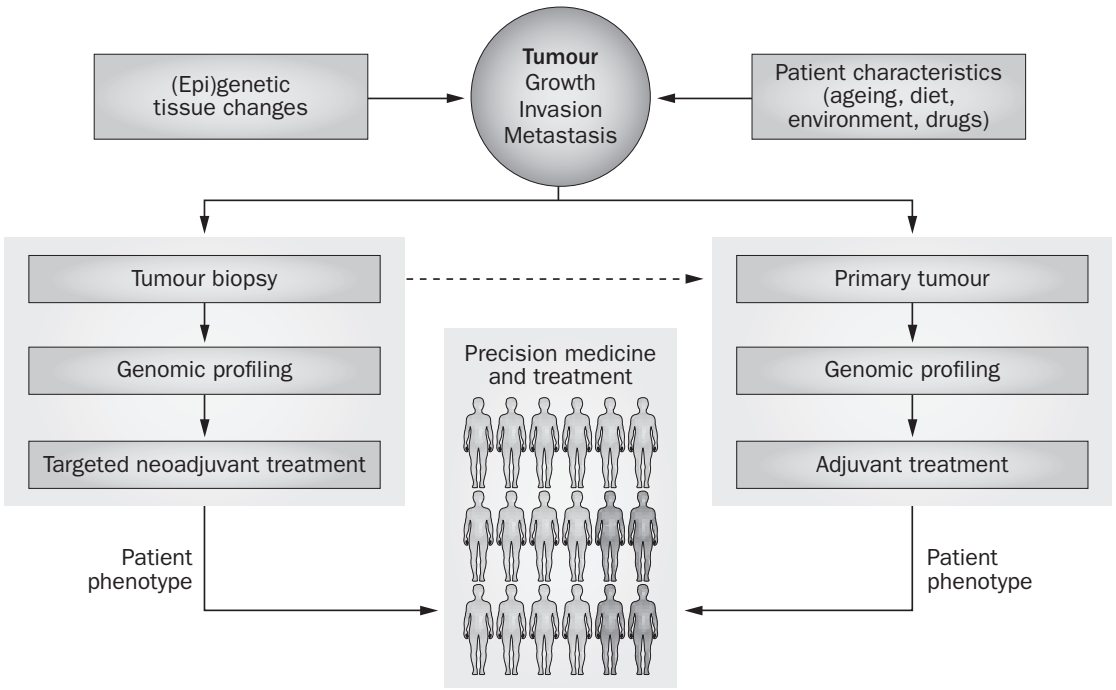


Figure 2: Global overview of the effect of genomic profiling on precision medicine.
This figure shows the effect of genomic profiling on precision medicine. (Epi)genetic tissue changes and patient characteristics influence tumor growth, invasion and metastasis. Genomic profiling can result in targeted neo-adjuvant treatment and adjuvant treatment through profiling of tumor biopsies or primary tumors consecutively, with the main goal of targeted treatment of the individual patient, better known as precision medicine. However, a patient's phenotype, for example, comorbidities, frailty and poly-pharmacy, must be taken into account for optimal targeted treatment and to reduce therapeutic morbidity, as written in the discussion session.

full ground in direct surgical decision-making. It might contribute to improved informed decision and better outcome, however, surgery still is, and will remain the most important cornerstone in cancer management.

REFERENCE LIST

(1) Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin* 2012;62:10-29.

(2) Amado RG, Wolf M, Peeters M et al. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* 2008;26:1626-1634.

(3) Armaghany T, Wilson JD, Chu Q, Mills G. Genetic alterations in colorectal cancer. *Gastrointest Cancer Res* 2012;5:19-27.

(4) Giardiello FM, Hamilton SR, Krush AJ et al. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N Engl J Med* 1993;328:1313-1316.

(5) Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57-70.

(6) Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646-674.

(7) Labayle D, Fischer D, Vielh P et al. Sulindac causes regression of rectal polyps in familial adenomatous polyposis. *Gastroenterology* 1991;101:635-639.

(8) Ladenheim J, Garcia G, Titzer D et al. Effect of sulindac on sporadic colonic polyps. *Gastroenterology* 1995;108:1083-1087.

(9) Balmana J, Diez O, Rubio I, Castiglione M. BRCA in breast cancer: ESMO Clinical Practice Guidelines. *Ann Oncol* 2010;21 Suppl 5:v20-v22.

(10) Albain KS, Barlow WE, Shak S et al. Prognostic and predictive value of the 21-gene recurrence score assay in postmenopausal women with node-positive, oestrogen-receptor-positive breast cancer on chemotherapy: a retrospective analysis of a randomised trial. *Lancet Oncol* 2010;11:55-65.

(11) Gray RG, Quirke P, Handley K et al. Validation study of a quantitative multigene reverse transcriptase-polymerase chain reaction assay for assessment of recurrence risk in patients with stage II colon cancer. *J Clin Oncol* 2011;29:4611-4619.

(12) van 't Veer LJ, Dai H, van de Vijver MJ et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002;415:530-536.

(13) Bild AH, Yao G, Chang JT et al. Oncogenic pathway signatures in human cancers as a guide to targeted therapies. *Nature* 2006;439:353-357.

(14) Bride MB, Neal L, Dilaveri CA et al. Factors associated with surgical decision making in women with early-stage breast cancer: a literature review. *J Womens Health (Larchmt)* 2013;22:236-242.

(15) Jatoi I. Options in breast cancer local therapy: who gets what? *World J Surg* 2012;36:1498-1502.

(16) Parry S, Win AK, Parry B et al. Metachronous colorectal cancer risk for mismatch repair gene mutation carriers: the advantage of more extensive colon surgery. *Gut* 2011;60:950-957.

(17) Romei C, Pardi E, Cetani F, Elisei R. Genetic and clinical features of multiple endocrine neoplasia types 1 and 2. *J Oncol* 2012;2012:705036.

(18) Duncan JA, Reeves JR, Cooke TG. BRCA1 and BRCA2 proteins: roles in health and disease. *Mol Pathol* 1998;51:237-247.

(19) Lord CJ, Ashworth A. Mechanisms of resistance to therapies targeting BRCA-mutant cancers. *Nat Med* 2013;19:1381-1388.

(20) Metcalfe K, Gershman S, Lynch HT et al. Predictors of contralateral breast cancer in BRCA1 and BRCA2 mutation carriers. *Br J Cancer* 2011;104:1384-1392.

(21) Miki Y, Swensen J, Shattuck-Eidens D et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 1994;266:66-71.

(22) Robson M, Svahn T, McCormick B et al. Appropriateness of breast-conserving treatment of breast carcinoma in women with germline mutations in BRCA1 or BRCA2: a clinic-based series. *Cancer* 2005;103:44-51.

(23) Neuburger J, Macneill F, Jeevan R, van der Meulen JH, Cromwell DA. Trends in the use of bilateral mastectomy in England from 2002 to 2011: retrospective analysis of hospital episode statistics. *BMJ Open* 2013;3.

(24) Rebbeck TR, Friebel T, Lynch HT et al. Bilateral prophylactic mastectomy reduces breast cancer risk in BRCA1 and BRCA2 mutation carriers: the PROSE Study Group. *J Clin Oncol* 2004;22:1055-1062.

(25) Metcalfe KA, Birenbaum-Carmeli D, Lubinski J et al. International variation in rates of uptake of preventive options in BRCA1 and BRCA2 mutation carriers. *Int J Cancer* 2008;122:2017-2022.

(26) Finch A, Beiner M, Lubinski J et al. Salpingo-oophorectomy and the risk of ovarian, fallopian tube, and peritoneal cancers in women with a BRCA1 or BRCA2 Mutation. *JAMA* 2006;296:185-192.

(27) Hermsen BB, Olivier RI, Verheijen RH et al. No efficacy of annual gynaecological screening in BRCA1/2 mutation carriers; an observational follow-up study. *Br J Cancer* 2007;96:1335-1342.

(28) Saha D, Roman C, Beauchamp RD. New strategies for colorectal cancer prevention and treatment. *World J Surg* 2002;26:762-766.

(29) Warrier SK, Kalady MF. Familial adenomatous polyposis: challenges and pitfalls of surgical treatment. *Clin Colon Rectal Surg* 2012;25:83-89.

(30) Schwarzova L, Stekrova J, Florianova M et al. Novel mutations of the APC gene and genetic consequences of splicing mutations in the Czech FAP families. *Fam Cancer* 2013;12:35-42.

(31) Church J, Burke C, McGannon E, Pastean O, Clark B. Predicting polyposis severity by proctoscopy: how reliable is it? *Dis Colon Rectum* 2001;44:1249-1254.

(32) Bulow S, Bulow C, Vasen H, Jarvinen H, Bjork J, Christensen IJ. Colectomy and ileorectal anastomosis is still an option for selected patients with familial adenomatous polyposis. *Dis Colon Rectum* 2008;51:1318-1323.

(33) Gunther K, Braunrieder G, Bittorf BR, Hohenberger W, Matzel KE. Patients with familial adenomatous polyposis experience better bowel function and quality of life after ileorectal anastomosis than after ileoanal pouch. *Colorectal Dis* 2003;5:38-44.

(34) Olsen KO, Juul S, Bulow S et al. Female fecundity before and after operation for familial adenomatous polyposis. *Br J Surg* 2003;90:227-231.

(35) Slors FJ, van Zuijlen PP, van Dijk GJ. Sexual and bladder dysfunction after total mesorectal excision for benign diseases. *Scand J Gastroenterol Suppl* 2000;48-51.

(36) Markowitz SD, Bertagnolli MM. Molecular origins of cancer: Molecular basis of colorectal cancer. *N Engl J Med* 2009;361:2449-2460.

(37) Martin-Lopez JV, Fishel R. The mechanism of mismatch repair and the functional analysis of mismatch repair defects in Lynch syndrome. *Fam Cancer* 2013;12:159-168.

(38) Engel C, Loeffler M, Steinke V et al. Risks of less common cancers in proven mutation carriers with lynch syndrome. *J Clin Oncol* 2012;30:4409-4415.

(39) Kohlmann W, Gruber SB. Lynch Syndrome. 1993.

(40) Kloos RT, Eng C, Evans DB et al. Medullary thyroid cancer: management guidelines of the American Thyroid Association. *Thyroid* 2009;19:565-612.

(41) Kurzrock R, Atkins J, Wheler J et al. Tumor marker and measurement fluctuations may not reflect treatment efficacy in patients with medullary thyroid carcinoma on long-term RET inhibitor therapy. *Ann Oncol* 2013.

(42) Rivkees SA, Mazzaferri EL, Verburg FA et al. The treatment of differentiated thyroid cancer in children: emphasis on surgical approach and radioactive iodine therapy. *Endocr Rev* 2011;32:798-826.

(43) Elisei R, Romei C, Renzini G et al. The timing of total thyroidectomy in RET gene mutation carriers could be personalized and safely planned on the basis of serum calcitonin: 18 years experience at one single center. *J Clin Endocrinol Metab* 2012;97:426-435.

(44) Xing M, Haugen BR, Schlumberger M. Progress in molecular-based management of differentiated thyroid cancer. *Lancet* 2013;381:1058-1069.

(45) Beer DG, Kardia SL, Huang CC et al. Gene-expression profiles predict survival of patients with lung adenocarcinoma. *Nat Med* 2002;8:816-824.

(46) Paik S, Shak S, Tang G et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *N Engl J Med* 2004;351:2817-2826.

(47) Paik S, Tang G, Shak S et al. Gene expression and benefit of chemotherapy in women with node-negative, estrogen receptor-positive breast cancer. *J Clin Oncol* 2006;24:3726-3734.

(48) Dowsett M, Cuzick J, Wale C et al. Prediction of risk of distant recurrence using the 21-gene recurrence score in node-negative and node-positive postmenopausal patients with breast cancer treated with anastrozole or tamoxifen: a TransATAC study. *J Clin Oncol* 2010;28:1829-1834.

(49) Mamounas EP, Tang G, Fisher B et al. Association between the 21-gene recurrence score assay and risk of locoregional recurrence in node-negative, estrogen receptor-positive breast cancer: results from NSABP B-14 and NSABP B-20. *J Clin Oncol* 2010;28:1677-1683.

(50) Glas AM, Floore A, Delahaye LJ et al. Converting a breast cancer microarray signature into a high-throughput diagnostic test. *BMC Genomics* 2006;7:278.

(51) van de Vijver MJ, He YD, van't Veer LJ et al. A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 2002;347:1999-2009.

(52) Arpino G, Generali D, Sapino A et al. Gene expression profiling in breast cancer: a clinical perspective. *Breast* 2013;22:109-120.

(53) Cardoso F, Van't Veer L, Rutgers E, Loi S, Mook S, Piccart-Gebhart MJ. Clinical application of the 70-gene profile: the MINDACT trial. *J Clin Oncol* 2008;26:729-735.

(54) O'Connell MJ, Lavery I, Yothers G et al. Relationship between tumor gene expression and recurrence in four independent studies of patients with stage II/III colon cancer treated with surgery alone or surgery plus adjuvant fluorouracil plus leucovorin. *J Clin Oncol* 2010;28:3937-3944.

(55) Salazar R, Roepman P, Capella G et al. Gene expression signature to improve prognosis prediction of stage II and III colorectal cancer. *J Clin Oncol* 2011;29:17-24.

(56) Maak M, Simon I, Nitsche U et al. Independent Validation of a Prognostic Genomic Signature (ColoPrint) for Patients With Stage II Colon Cancer. *Ann Surg* 2013.

(57) Ayers M, Symmans WF, Stec J et al. Gene expression profiles predict complete pathologic response to neoadjuvant paclitaxel and fluorouracil, doxorubicin, and cyclophosphamide chemotherapy in breast cancer. *J Clin Oncol* 2004;22:2284-2293.

(58) Chang JC, Wooten EC, Tsimelzon A et al. Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer. *Lancet* 2003;362:362-369.

(59) Cho JH, Park JM, Park HS, Park S, Kim SI, Park BW. Oncologic safety of breast-conserving surgery compared to mastectomy in patients receiving neoadjuvant chemotherapy for locally advanced breast cancer. *J Surg Oncol* 2013.

(60) Shin HC, Han W, Moon HG et al. Breast-conserving surgery after tumor downstaging by neoadjuvant chemotherapy is oncologically safe for stage III breast cancer patients. *Ann Surg Oncol* 2013; 20:2582-2589.

(61) Rose JB, Rocha FG, Alseidi A et al. Extended Neoadjuvant Chemotherapy for Borderline Resectable Pancreatic Cancer Demonstrates Promising Postoperative Outcomes and Survival. *Ann Surg Oncol* 2014.

(62) Hannemann J, Oosterkamp HM, Bosch CA et al. Changes in gene expression associated with response to neoadjuvant chemotherapy in breast cancer. *J Clin Oncol* 2005;23:3331-3342.

(63) Hartley A, Ho KF, McConkey C, Geh JI. Pathological complete response following pre-operative chemoradiotherapy in rectal cancer: analysis of phase II/III trials. *Br J Radiol* 2005;78:934-938.

(64) Habr-Gama A, de Souza PM, Ribeiro U, Jr. et al. Low rectal cancer: impact of radiation and chemotherapy on surgical treatment. *Dis Colon Rectum* 1998;41:1087-1096.

(65) Habr-Gama A, Perez RO, Nadalin W et al. Operative versus nonoperative treatment for stage 0 distal rectal cancer following chemoradiation therapy: long-term results. *Ann Surg* 2004;240:711-717.

(66) Habr-Gama A, Perez RO, Nadalin W et al. Long-term results of preoperative chemoradiation for distal rectal cancer correlation between final stage and survival. *J Gastrointest Surg* 2005;9:90-99.

(67) Habr-Gama A, Perez RO, Proscurshim I et al. Patterns of failure and survival for nonoperative treatment of stage c0 distal rectal cancer following neoadjuvant chemoradiation therapy. *J Gastrointest Surg* 2006;10:1319-1328.

(68) Habr-Gama A. Assessment and management of the complete clinical response of rectal cancer to chemoradiotherapy. *Colorectal Dis* 2006;8 Suppl 3:21-24.

(69) Maas M, Beets-Tan RG, Lambregts DM et al. Wait-and-see policy for clinical complete responders after chemoradiation for rectal cancer. *J Clin Oncol* 2011;29:4633-4640.

(70) Smith FM, Wiland H, Mace A, Pai RK, Kalady MF. Clinical criteria underestimate complete pathological response in rectal cancer treated with neoadjuvant chemoradiotherapy. *Dis Colon Rectum* 2014;57:311-315.

(71) van Hagen P, Hulshof MC, van Lanschot JJ et al. Preoperative chemoradiotherapy for esophageal or junctional cancer. *N Engl J Med* 2012;366:2074-2084.

(72) Kim JY, Hofstetter WL. Esophagectomy after chemoradiation: who and when to operate. *Semin Thorac Cardiovasc Surg* 2012;24:288-293.

(73) Stahl M, Stuschke M, Lehmann N et al. Chemoradiation with and without surgery in patients with locally advanced squamous cell carcinoma of the esophagus. *J Clin Oncol* 2005;23:2310-2317.

(74) Furlong H, Bass G, Breathnach O, O'Neill B, Leen E, Walsh TN. Targeting therapy for esophageal cancer in patients aged 70 and over. *J Geriatr Oncol* 2013;4:107-113.

(75) Edgren G, Adami HO, Weiderpass E, Nyren O. A global assessment of the oesophageal adenocarcinoma epidemic. *Gut* 2013;62:1406-1414.

(76) Hong MK, Sapre N, Phal PM et al. Percutaneous image-guided biopsy of prostate cancer metastases yields samples suitable for genomics and personalised oncology. *Clin Exp Metastasis* 2013.

(77) Marshall D, Laberge JM, Firetag B, Miller T, Kerlan RK. The changing face of percutaneous image-guided biopsy: molecular profiling and genomic analysis in current practice. *J Vasc Interv Radiol* 2013;24:1094-1103.

(78) Al-Leswas D, O'Reilly DA, Poston GJ. Biopsy of solid liver tumors: adverse consequences. *Hepatobiliary Pancreat Dis Int* 2008;7:325-327.

(79) Boutin C, Rey F, Viallat JR. Prevention of malignant seeding after invasive diagnostic procedures in patients with pleural mesothelioma. A randomized trial of local radiotherapy. *Chest* 1995;108:754-758.

(80) Jones OM, Rees M, John TG, Bygrave S, Plant G. Biopsy of resectable colorectal liver metastases causes tumor dissemination and adversely affects survival after liver resection. *Br J Surg* 2005;92:1165-1168.

(81) Liebens F, Carly B, Cusumano P et al. Breast cancer seeding associated with core needle biopsies: a systematic review. *Maturitas* 2009;62:113-123.

(82) Mesker WE, Junggeburt JM, Szuhai K et al. The carcinoma-stromal ratio of colon carcinoma is an independent factor for survival compared to lymph node status and tumor stage. *Cell Oncol* 2007; 29:387-398.
(83) Varga Z, Sinn P, Fritzsche F et al. Comparison of EndoPredict and Oncotype DX test results in hormone receptor positive invasive breast cancer. *PLoS One* 2013;8:e58483.
(84) McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. REporting recommendations for tumor MARKer prognostic studies (REMARK). *Breast Cancer Res Treat* 2006;100:229-235.
(85) Glynne-Jones R, Hughes R. Critical appraisal of the 'wait and see' approach in rectal cancer for clinical complete responders after chemoradiation. *Br J Surg* 2012;99:897-909.
(86) de Campos-Lobato LF, Geisler DP, da Luz MA, Stocchi L, Dietz D, Kalady MF. Neoadjuvant therapy for rectal cancer: the impact of longer interval between chemoradiation and surgery. *J Gastrointest Surg* 2011;15:444-450.
(87) Tulchinsky H, Shmueli E, Figer A, Klausner JM, Rabau M. An interval >7 weeks between neoadjuvant therapy and surgery improves pathologic complete response and disease-free survival in patients with locally advanced rectal cancer. *Ann Surg Oncol* 2008;15:2661-2667.
(88) Ruol A, Rizzetto C, Castoro C et al. Interval between neoadjuvant chemoradiotherapy and surgery for squamous cell carcinoma of the thoracic esophagus: does delayed surgery have an impact on outcome? *Ann Surg* 2010;252:788-796.
(89) Buchholz TA, Stivers DN, Stec J et al. Global gene expression changes during neoadjuvant chemotherapy for human breast cancer. *Cancer J* 2002;8:461-468.
(90) Zujewski JA, Kamin L. Trial assessing individualized options for treatment for breast cancer: the TAILORx trial. *Future Oncol* 2008;4:603-610.
(91) Bernards R. A missing link in genotype-directed cancer therapy. *Cell* 2012;151:465-468.
(92) http://globocan.iarc.fr. 2014.
(93) Baylin SB, Jones PA. A decade of exploring the cancer epigenome - biological and translational implications. *Nat Rev Cancer* 2011;11:726-734.
(94) Goel A, Boland CR. Epigenetics of colorectal cancer. *Gastroenterology* 2012;143:1442-1460.
(95) Sharma S, Kelly TK, Jones PA. Epigenetics in cancer. *Carcinogenesis* 2010;31:27-36.
(96) Arnold CN, Goel A, Boland CR. Role of hMLH1 promoter hypermethylation in drug resistance to 5-fluorouracil in colorectal cancer cell lines. *Int J Cancer* 2003;106:66-73.
(97) Baylin SB. Resistance, epigenetics and the cancer ecosystem. *Nat Med* 2011;17:288-289.
(98) Huynh KT, Chong KK, Greenberg ES, Hoon DS. Epigenetics of estrogen receptor-negative primary breast cancer. *Expert Rev Mol Diagn* 2012;12:371-382.
(99) Lyko F, Brown R. DNA methyltransferase inhibitors and the development of epigenetic cancer therapies. *J Natl Cancer Inst* 2005;97:1498-1506.
(100) Sekeres MA, Tiu RV, Komrokji R et al. Phase 2 study of the lenalidomide and azacitidine combination in patients with higher-risk myelodysplastic syndromes. *Blood* 2012;120:4945-4951.
(101) Garcia-Manero G. Demethylating agents in myeloid malignancies. *Curr Opin Oncol* 2008;20:705-710.
(102) Huang Y, Nayak S, Jankowitz R, Davidson NE, Oesterreich S. Epigenetics in breast cancer: what's new? *Breast Cancer Res* 2011;13:225.
(103) Grady WM, Rajput A, Lutterbaugh JD, Markowitz SD. Detection of aberrantly methylated hMLH1 promoter DNA in the serum of patients with microsatellite unstable colon cancer. *Cancer Res* 2001;61:900-902.
(104) Hellebrekers DM, Lentjes MH, van den Bosch SM et al. GATA4 and GATA5 are potential tumor suppressors and biomarkers in colorectal cancer. *Clin Cancer Res* 2009;15:3990-3997.

(105) Nagasaka T, Tanaka N, Cullings HM et al. Analysis of fecal DNA methylation to detect gastrointestinal neoplasia. *J Natl Cancer Inst* 2009;101:1244-1258.
(106) Ouyang DL, Chen JJ, Getzenberg RH, Schoen RE. Noninvasive testing for colorectal cancer: a review. *Am J Gastroenterol* 2005;100:1393-1403.
(107) Heyn H, Esteller M. DNA methylation profiling in the clinic: applications and challenges. *Nat Rev Genet* 2012;13:679-692.
(108) Goessl C, Krause H, Muller M et al. Fluorescent methylation-specific polymerase chain reaction for DNA-based detection of prostate cancer in bodily fluids. *Cancer Res* 2000;60:5941-5945.
(109) Sunami E, Shinozaki M, Higano CS et al. Multimarker circulating DNA assay for assessing blood of prostate cancer patients. *Clin Chem* 2009;55:559-567.
(110) Yegnasubramanian S, Kowalski J, Gonzalgo ML et al. Hypermethylation of CpG islands in primary and metastatic human prostate cancer. *Cancer Res* 2004;64:1975-1986.
(111) Nakayama H, Hibi K, Takase T et al. Molecular detection of p16 promoter methylation in the serum of recurrent colorectal cancer patients. *Int J Cancer* 2003;105:491-493.
(112) Yamaguchi S, Asao T, Nakamura J, Ide M, Kuwano H. High frequency of DAP-kinase gene promoter methylation in colorectal cancer specimens and its identification in serum. *Cancer Lett* 2003;194: 99-105.
(113) Tan SH, Ida H, Lau QC et al. Detection of promoter hypermethylation in serum samples of cancer patients by methylation-specific polymerase chain reaction for tumor suppressor genes including RUNX3. *Oncol Rep* 2007;18:1225-1230.
(114) Ebert MP, Model F, Mooney S et al. Aristaless-like homeobox-4 gene methylation is a potential marker for colorectal adenocarcinomas. *Gastroenterology* 2006;131:1418-1430.
(115) Lofton-Day C, Model F, Devos T et al. DNA methylation biomarkers for blood-based colorectal cancer screening. *Clin Chem* 2008;54:414-423.

CHAPTER 10

Summary and General discussion

SUMMARY AND GENERAL DISCUSSION

Over the past decades, major advances have been made in the treatment of CRC patients. The introduction of new surgical techniques and (neo) adjuvant therapies has greatly improved clinical outcome in CRC patients. A great example is the introduction of the total mesorectal excision (TME) technique and pre-operative radiotherapy in rectal cancer, which decreased the local recurrence rate from 11 to 6% [1]. In colon cancer, the introduction of adjuvant chemotherapy with fluorouracil and levamisole greatly reduced the mortality rate by 33% among stage III patients [2]. The addition of oxaliplatin to this regimen further improved clinical outcome in stage II and III colon cancer patients with a three years disease-free survival of 78% in the MOSAIC trial [3]. Final results of this trial reporting on 5-year disease-free survival and 6-year overall survival also proved that adding oxaliplatin to fluorouracil and levamisole was associated with survival benefits. However, significant difference in survival between these two regimens was lost in stage II colon cancer patients [4]. Therefore, the role and benefit of adjuvant chemotherapy in stage II colon cancer patients still remains controversial [4;5]. Altogether, this has led to current recommendations in the Netherlands where patients with stage III and high-risk stage II colon cancer, e.g. those with T4 tumor extent or vascular invasion, are offered adjuvant chemotherapy with the FOLFOX regimen, consisting of oxaliplatin, fluorouracil and leucovorin [3].

In addition to stage II colon cancer patients, the role of adjuvant chemotherapy in rectal cancer remains debatable as well. Up till now, studies have failed to show significant survival benefits for adjuvant chemotherapy in rectal cancer patients, who are, according to current guidelines, treated with preoperative radiotherapy [3;6-8]. Adjuvant chemotherapy in rectal cancer is therefore not implemented in daily clinical practice in the Netherlands.

Even though major advances in treatment of CRC have been made, mortality still remains high. In the Netherlands, each year approximately 9000 patients are diagnosed with CRC and 4000 deaths occur as a consequence of this disease (www.cijfersoverkanker.nl). Morbidity associated with current treatments should not be underestimated as well. For example, studies in rectal cancer have evaluated the short- and long term morbidity of radiotherapy, where preoperative radiotherapy was associated with faecal incontinence, urgency, anal blood loss and sexual dysfunction [9]. A significant number of (neo)adjuvant treated patients will not show any treatment benefit or not even need treatment to increase prognosis, and approximately 30% of stage II colon cancer patients suffer from recurrent disease within 5 years after surgery [10]. Nowadays, prognostication and treatment allocation are majorly influenced by tumor location and tumor stage (TNM). However, tumor classification has become more complex over the past years since the TNM staging system failed to provide clinicians with the optimal staging

tool it was designed for. Patient survival varies widely within each stage and positive lymph nodes, which determine tumor stage, are easily missed in routine pathological assessment. Under-treatment and over-treatment of some patients exists when using this system for treatment allocation [11-14]. Therefore, the use of TNM stage falls short in daily clinical practice and needs to be supplemented with additional biomarkers that can improve current staging and treatment allocation criteria substantially. Predicting the clinical behavior of a tumor through a combination of clinical, pathological and biological characteristics might lead to a well-targeted treatment in the individual patient, thereby increasing treatment benefit and limiting negative side effects. In this thesis we therefore evaluated prognostic and predictive biomarkers in CRC for improved risk stratification and treatment benefit in the individual patient, with the introduction of precision medicine in the near future as ultimate goal. This thesis is divided in three parts. In ***Part one*** we investigated biomarkers related to important hallmarks of cancer, which were able to adequately assess prognosis in CRC patients. In ***Part two*** we established a survival benefit in colon cancer patients treated with low dose aspirin after diagnosis and investigated predictive biomarkers, which were able to predict which patients would benefit from aspirin treatment after a colon cancer diagnosis. Finally, in ***Part three*** we discussed the use of prognostic and predictive biomarkers in clinical practice, its utility and the road to precision medicine.

PART ONE: PROGNOSTIC BIOMARKERS IN COLORECTAL CANCER

In 2000, Hanahan and Weinberg published an important article about ' the hallmarks of cancer', which are six biological capabilities tumors have to acquire during the multistep development of human cancers. These hallmarks are sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death [15]. In 2011, they added two emerging hallmarks; reprogramming of energy metabolism and evading immune recognition and recognized the importance of the tumor-microenvironment in tumor development. The hallmarks constitute an organizing principle for rationalizing the complexities of neoplastic disease. Recognition of these hallmarks will increasingly affect prognostication and the development of new means to treat human cancer [15]. In this part we investigated biomarkers related to some of these hallmarks, such as sustaining proliferative signaling, resisting cell death and evading immune recognition.

The last decades, research has indicated a substantial influence of the immune system on tumor growth, which showed to be both tumor suppressing and promoting [16]. In **Chapter 2 and 3** we investigated the prognostic value of important immune recognition evading mechanisms in colon cancer and in rectal cancer separately by analyzing

HLA class I tumor expression, tumor expression of non-classical HLA class I molecules (HLA-E and HLA-G) and tumor infiltration with immunosuppressive regulatory T cells (Tregs). The goal of these studies was to establish a tumor profile based on biomarkers that reflect a tumor's immune susceptibility status and to determine its relationship to patient outcome.

In 285 colon cancer patients (**Chapter 2**), loss of HLA class I was significantly associated with a better overall survival and disease-free survival, which could be explained by elimination of tumor cells by natural killer (NK) cells once these tumor cells metastasize to the bloodstream [17-19]. When the immune markers were combined, three distinct survival patterns based on immune surveillance were identified. Patients with tumors showing loss of HLA class I and negative HLA-E and –G expression, irrespective of Treg tumor infiltration, showed the best prognosis. Absence of HLA-E and -G expression possibly made these tumors, who have lost their HLA class I expression, even more susceptible to NK cell elimination, further explaining their favorable prognosis [20;21]. In contrast, patients showing the worst prognosis were patients with tumors with HLA class I downregulation and low Treg infiltration, irrespective of HLA-E and –G expression. Since tumors are thought to be 'immunoedited' through a Darwinian selection process into poorly immunogenic tumor cell variants invisible to the immune system [16], we hypothesized that these poorly immune-recognized tumors are already edited by Cytotoxic T-cells (CTL), because they partly lost their HLA class I expression. Consequently, these tumors will elicit a minimal CTL attack, resulting in tumor progression. The absence of Tregs in the tumor micro-environment of these tumors further strengthens our hypothesis. Because of the opposing actions of Tregs and CTL in tumor immunity, Tregs will not be needed for immune escape when CTL presence is scarce [22]. In summary, this study showed a complex and multifaceted interplay between different immune escape mechanisms, highlighting the need for combined immune marker analysis to better reflect patient outcome. We were able to determine three distinct survival patterns in colon cancer based on immune surveillance (Figure 1), which represented significant independent clinical prognostic value in colon cancer patients.

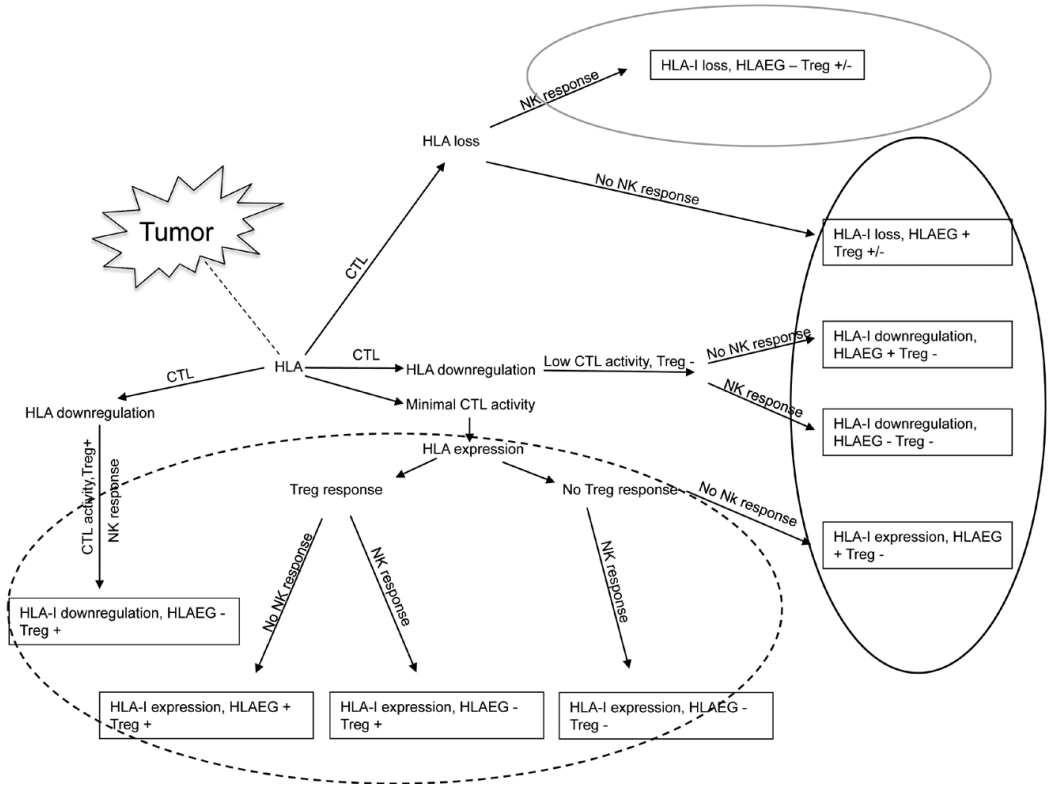


Figure 1: Global overview of immune escape mechanisms based on literature and results we established in a cohort of 285 colon cancers in which HLA class I tumor expression, HLA-E tumor expression, HLA-G tumor expression and Treg infiltration were investigated

The tumors with a certain phenotype in the gray, dashed and black circle indicate tumors that are high, intermediate or low immune susceptible with a good, intermediate and worse prognosis respectively. Treg, immunosuppressive regulatory T cell; CTL, Cytotoxic T cell; NK, natural killer cell.

In **Chapter 3**, we investigated the prognostic relevance of the same immune markers, independently and combined, in 495 rectal cancer patients. In this study, HLA class I tumor expression and a high Treg tumor infiltration were related to a better clinical outcome in these rectal cancer patients. Interestingly, strong HLA-G expression was also significantly related to a better survival. These results are remarkable since HLA-G expression can inhibit NK cells from lysing tumor cells that have lost or downregulated classical HLA class I expression as a secondary immune escape [23;24]. The reason for this seemingly opposing effect of HLA-G expression remains unclear. Immune regulation in cancer still remains complex and multifaceted, and not all immune-related mechanisms are completely clear. Possibly, HLA-G expression does not play an influential role in rectal cancer when HLA class I expression is still present.

When the immune markers were combined, again three distinct patterns in patient survival based on immune surveillance were identified. Prognosis increased with a decrease in negative prognostic markers, thus patients with tumors bearing two or three negative prognostic markers, e.g. loss of HLA class I tumor expression, weak HLA-G

tumor expression and low tumor infiltration with Tregs, showed a worse prognosis and therefore qualified as very low immune susceptible. Furthermore, patients with tumors showing loss of HLA class I expression, low Treg infiltration and strong HLA-G expression showed the worst outcome perspectives. We hypothesized that these patients probably had tumors which were highly 'immunoedited', since these tumors have lost their HLA class I expression, causing a minimal CTL attack and subsequently attracted little to no Tregs. Because of strong HLA-G expression they probably were able to escape further immune recognition through inhibition of NK cell recognition [23;24]. Interestingly, in contrast to what we have reported above, HLA-G expression is in this subset of poorly immune-recognized tumors associated with a worse survival. HLA-G expression might only play an influential role during this phase of 'immuoediting' as second immune escape mechanism, when HLA class I expression has already been lost.

These two chapters have provided us with some confusing and opposing results, as, compared to colon cancer, some different immune escape mechanisms seem to occur in rectal cancer. In colon cancer, loss of HLA class I was significantly related to a better survival. In rectal cancer, best survival outcomes were seen for patients with tumors showing expression of HLA class I. This might suggest biological differences between colon and rectal tumors. One of these biological differences might be the microsatellite status of the tumor. Approximately 50% of all proximal colon tumors show microsatellite instability (MSI), whereas almost all distal colon and rectal cancers are microsatellite stable (MSS) tumors [25;26]. MSI has been associated with loss of HLA class I as well as a better prognosis, possibly influencing prognostic results when analyzing HLA class I in colorectal tumors [27;28]. Unfortunately, in our colon cancer cohort the number of MSI tumors that was successfully determined was too small to perform separate analyses in MSI and MSS tumors.

When all immune markers were combined, differences in immune escape mechanisms became even clearer. In colon cancer, patients with tumors showing loss of HLA class I and negative HLA-E and -G expression, irrespective of Treg infiltration, were related to a better survival. In contrast, tumors with the same characteristics were related to a worse outcome in rectal cancer. Again, microsatellite status might influence these results.

Recently, the Cancer Genome Atlas Network investigated biological differences between colon and rectal cancer, but only established differences in anatomical tumor site with more hypermethylation in right-sided tumors, possibly explained by different embryonic origins of right-and left-sided tumors [29]. Therefore, the question still remains if there are true biological differences between colon and rectal cancer and further studies should focus on separate analyses of these tumors.

In **Chapter 4**, we performed a combined analysis of biomarkers of proliferation and apoptosis in colon cancer, namely Ki67 and cleaved caspase-3. A key factor in tissue

homeostasis, especially of the intestinal mucosa, is a balance between the level of cell death and cell proliferation [30-32]. Disturbance of this balance could contribute to initiation and maintenance of tumor growth and development [15;33]. Previous studies in CRC showed contradicting results with respect to the association between apoptosis and proliferation in tumor resection specimens and patient outcome, especially when comparing tumors originating from the colon and rectum [32;34-39]. Also, the prognostic value of apoptosis and proliferation seems to be influenced by tumor location and microsatellite status [37;40;41].

The contradicting results derived from these studies strengthened our hypothesis that a balance between both these processes determines patient's clinical outcome. Our study showed that a combined analysis of the level of tumor cell proliferation and apoptosis was significantly related to patient outcome in 285 stage I-IV colon cancer patients with respect to disease-free survival and overall survival. Patients with a strong proliferation and presence of apoptosis in their tumors showed the best survival outcomes. Interestingly, the impact of this combined analysis of proliferation and apoptosis on patient outcome varied with tumor location and therefore highly likely with tumor microsatellite status, since significantly more MSI tumors were located on the right side of the colon. Unfortunately, the number of MSI tumors in our cohort was too small to perform stratified survival analysis for microsatellite status.

In the left-sided cohort the patients with a balance between proliferation and apoptosis in their tumors performed better with respect to outcome. As you would expect from high proliferative tumors, patients with left-sided tumors showing high proliferation levels and absence of apoptosis had the worst outcome perspectives. In contrast, right-sided tumors with high proliferation levels and absence of apoptosis performed significantly better. Based on these results we hypothesized that it is either tumor microsatellite status or tumor location, which influences the prognostic value of the balance between tumor cell proliferation and apoptosis. It is not unlikely that the tumor microsatellite status influences the balance between tumor cell proliferation and apoptosis. MSI tumors are known to have high levels of proliferation and tend to accumulate gene mutations leading to increased production of abnormal peptides [40;41]. This might result in an immune reaction leading to higher levels of apoptosis, which possibly explains the favorable prognosis of patients with right-sided tumors showing high proliferation levels [42]. However, further studies investigating these two important hallmarks are necessary and should focus on separate analyses of colon- and rectal cancers, where tumor microsatellite status and location are be taken into account as well.

In **Chapter 5**, we performed a validation of the 12-gene Colon Cancer Recurrence Score® Assay as a predictor of recurrence risk in stage II and III rectal cancer patients treated with surgery alone from the Dutch TME trial [1]. The Onco*type* DX Colon Cancer Recurrence

Score (RS) (Genomic Health, Redwood City, CA, USA) was developed by using tumor gene expression data from 1851 patients with resected colon cancer from four independent clinical trials [43]. This was followed by the design of the 12-gene colon cancer Recurrence Score (RS), which was validated in the QUASAR clinical trial beyond other clinical covariates [44]. Predefined risk groups were categorized as low, intermediate or high risk for tumor recurrence according to patients' RS values, which gave the possibility to specifically allocate cancer patients for (adjuvant) treatment regimens. In this validation study performed in rectal cancer, RS predicted risk of recurrence, risk of distant recurrence, and rectal cancer-specific survival. The effect of RS was most prominent in stage II rectal cancer and attenuated with more advanced stage. RS may be clinically useful in stage II rectal cancer patients, where RS can help identify high-risk patients who could benefit from -- and low-risk patients who may forego -- adjuvant chemotherapy (Figure 2).

Up till now trials failed to show a survival benefit with adjuvant chemotherapy for pre-operatively treated rectal cancer patients [6-8]. However, efforts are underway to study reduced-intensity approaches, including those that spare radiation or even surgery. Incorporation of the Recurrence Score assay into clinical trials, such as the TAILORx and RxPonder trials in breast cancer [45;46], may enable these efforts through improved patient stratification for risk-adapted treatment strategies.

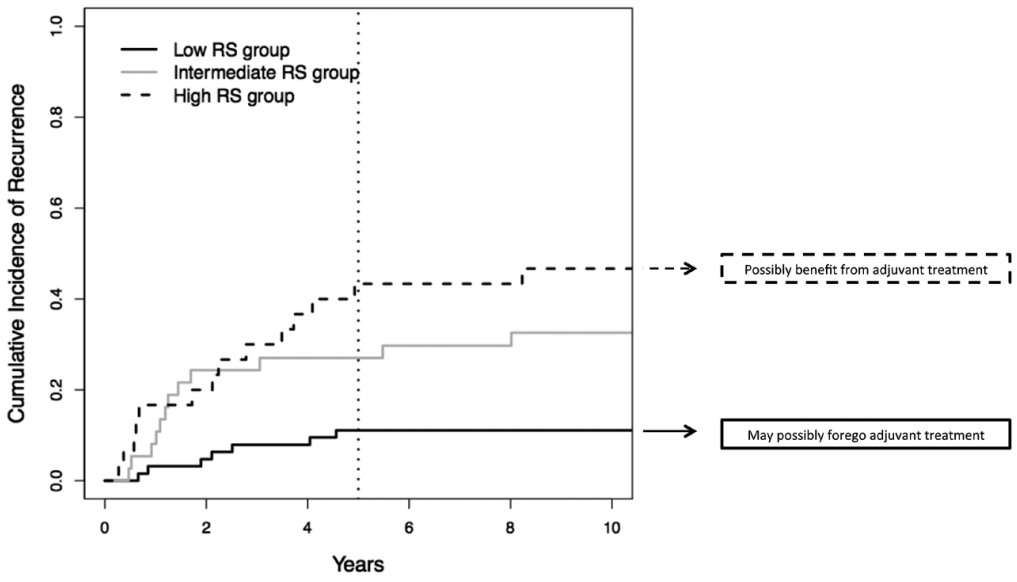


Figure 2: Cumulative incidence of recurrence in 297 rectal cancer patients.
Predefined risk groups were categorized as low, intermediate or high risk for tumor recurrence according to patients' Recurrence Score (RS) values based on the 12-gene Colon Cancer Recurrence Score® Assay, giving the opportunity to specifically allocate adjuvant treatment in the individual patient. This figure is derived from Reimers *et al*., Validation of the 12-gene Colon Cancer Recurrence Score as a predictor of recurrence risk in stage II and III rectal cancer patient, J Natl Cancer Inst 2014 Sep 26:106(11)

PART TWO: TREATMENT OF COLON CANCER AND PREDICTIVE BIOMARKERS

Aspirin and other nonsteroidal anti -inflammatory drugs have shown to be effective in preventing CRC [47-49]. More recently, aspirin has also shown promising results when used after CRC diagnosis [50-52]. In **Chapter 6** we performed a subanalysis in elderly colon cancer patients of the cohort used by Bastiaannet *et al.* [50] to investigate the benefit of low-dose aspirin (80mg) treatment after diagnosis. Patients with rectal cancer were excluded from analysis as these patients did not show any benefit from aspirin treatment. In this study, aspirin use after diagnosis was significantly associated with an improved survival of 40% in older colon cancer patients (≥ 70 years of age) compared to nonusers. This study implicates that aspirin could be an effective adjuvant agent in the treatment of colon cancer, especially in older, chemo-naïve colon cancer patients. Demonstration of a significant therapeutic effect of a well-tolerated, inexpensive drug would be a major clinical advancement.

The exact mechanism by which aspirin exerts its anti-cancer effect still remains largely unknown. It might be that the anti-inflammatory and chemopreventive effects of aspirin are mediated through direct inhibition of COX-1 and COX-2 [53-55]. COX-1 is responsible for platelet aggregation through production of TXA2 in platelets [56]. COX-2 plays an important role in colorectal carcinogenesis, invasion, angiogenesis and metastasis [54] and approximately 70% of colorectal tumors express COX-2 [51;57]. Studies have shown that this COX-2 effect can be reversed by selective COX-2 inhibitors [54]. COX-2 independent pathways, such as suppression of IL-4, NF-κB, insulin-like growth factor 1 (IGF-1), and the inhibition of Wnt-signaling and stem cell growth possibly as the result of enhanced beta-catenin phosphorylation have also been described to contribute to the anti-cancer effects of aspirin [58-62]. Recently, several studies on aspirin benefit in CRC were performed on data from the Nurses' Health Study in the USA. First, Chan *et al*. reported a survival benefit for aspirin use after diagnosis in CRC patients, which seemed to be dependent on COX-2 expression of the tumor. A much lower risk of CRC-specific and overall mortality with tumors that overexpress COX-2 was found [51]. A second study of the same research group showed that the survival benefit from aspirin use after diagnosis was restricted to patients with mutant *PIK3CA* tumors. Patients with wild-type *PIK3CA* tumors did not benefit from aspirin treatment [63]. The phosphatidylinositol 3-kinase (PI3K) signaling pathway plays an important role in carcinogenesis [64]. Mutations in *PIK3CA* are present in approximately 15 to 20% of CRCs [65-67]. Up-regulation of PI3K enhances COX-2 activity and prostaglandin E2 synthesis, resulting in inhibition of apoptosis in colon-cancer cells [68]. Aspirin might suppress tumor development and induce apoptosis by blocking this PI3K pathway [69].

As it is desirable to reduce overtreatment of patients and lower incidental side effects of aspirin treatment, we also tried to find predictive biomarkers for aspirin treatment in

colon cancer. The metastatic potential of cancer cells that are shed into the bloodstream can be modified by environmental conditions, including platelets and bone marrow-derived cells in the vasculature [70]. As soon as cancer cells enter the bloodstream they interact with platelets [71]. Through tumor cell coating, platelets are thought to protect disseminating tumor cells from lysis by immune cells such as NK cells. Tumor cell coating leads to platelet activation and degranulation followed by release of a variety of factors capable of influencing NK reactivity [72]. The interaction between platelets and tumor cells is also thought to transfer HLA class I from the platelet onto the tumor cell surface resulting in a HLA class I-positive phenotype, or 'pseudoself'. This platelet-derived HLA class I blocks NK cell activity. Because platelet-derived HLA class I presents self-peptides, reflecting the normal ligandome of the megakaryocyte lineage, CTLs are not activated as well [72].

Aspirin influences platelet aggregation through COX-1 inhibition [56]. Most likely tumor cell coating and platelet-tumor cell interaction are affected as well. In case of aspirin use, tumor cells are now prone for lysis by immune cells. NK cells preferentially recognize and eliminate cells with low or absent expression of HLA class I [21;23], We therefore hypothesized that the survival benefit associated with low dose aspirin use after a cancer diagnosis would be associated with tumors that have low or absent HLA class I expression. In **Chapter 7** we showed that aspirin use after a colon cancer diagnosis was associated with improved survival if tumors expressed HLA class I on their cell surface, contrary to the original hypothesis. There are two possible explanations for this intriguing observation. First, the disruption of platelet aggregates with aspirin that shield HLA class I expressing, circulating tumor cells might make these cells more susceptible for T-cell mediated immune surveillance. Second, direct contact of platelets and tumor cells results in secretion of TGF-β and activation of the NF-κB pathway, which, in synergistic action, prime circulating tumor cells for subsequent metastases [70]. Aspirin might inhibit platelet-tumor cell signaling and prevents epithelial-mesenchymal transition in circulating tumor cells, thereby reducing the metastatic potential. HLA class I expression might be necessary for this platelet mediated NF-κB signaling in circulating tumor cells resulting in an epithelial-mesenchymal-like phenotype with enhanced metastatic potential (Figure 3).

Our data was not able to confirm the previously published results from the USA group, which demonstrated that the benefits of aspirin after a colorectal cancer diagnosis were associated with strong COX-2 expression in the original tumor and the presence of mutations in *PIK3CA* [51;63]. In our cohort, there was no difference in benefit from aspirin use after a colon cancer diagnosis when the survival analyses were stratified for COX-2 expression and *PIK3CA* mutation status. Interestingly, research performed by an English group recently confirmed the survival benefit of aspirin in *PIK3CA* mutated CRCs, however, the predictive value of COX-2 expression was again not validated in this cohort [73].

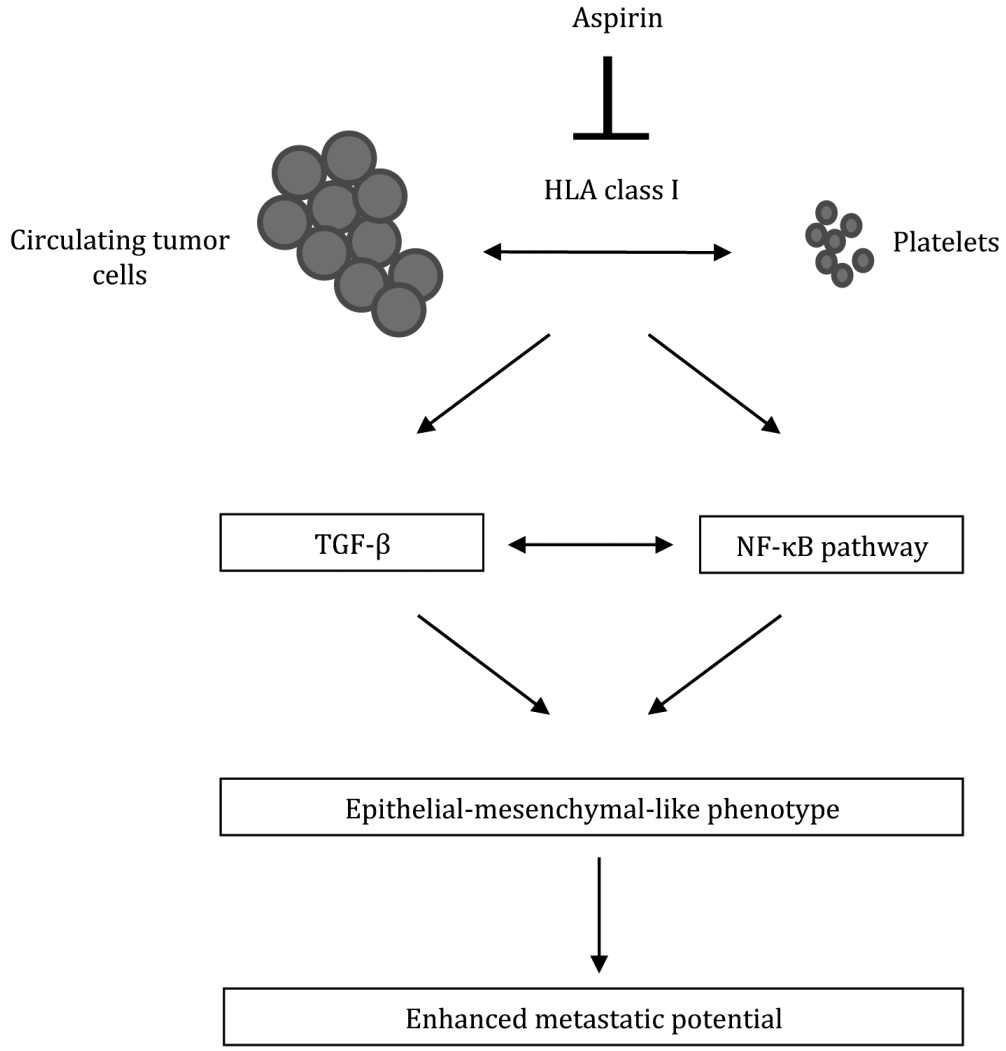


Figure 3:
In this model direct contact of platelets and tumor cells results in secretion of TGF-β and activation of the NF-κB pathway, which, in synergistic action, prime circulating tumor cells for subsequent metastases. Aspirin might inhibit platelet-tumor cell signaling (which is dependent upon intact HLA expression) and prevents epithelial-mesenchymal transition in circulating tumor cells, thereby reducing the metastatic potential.

The contradicting results might be pharmacologically explained, since different dosages of aspirin are investigated in these studies (USA group 325 mg, English group 100 mg, our group 80 mg). Data on aspirin indicate that systemic concentrations of aspirin reached with low-doses are inadequate to permanently acetylate COX-2, but are optimal for platelet inhibition [74]. This might explain why in our cohort, where low-dose aspirin was investigated, strong COX-2 expression and *PIK3CA* mutations were not validated as predictive biomarkers. Furthermore, there may be more than one mechanism of action that accounts for the anti-cancer effects of aspirin; a direct anti-platelet effect due to inhibition of COX-1, that is responsible for the reduction in metastases and only requires

a dose of aspirin that inhibits platelets; and a second mechanism activated with higher or more frequent dosing that inhibits the COX-2 pathway in systemic tissues.

Reflecting on the results derived from this thesis the apoptotic pathway could also be a potential field of interest for studying the anti-cancer effects of aspirin. Aspirin has shown to promote apoptosis, either through suppression of IL-4 gene expression, which is essential for the resistance to DNA damage-induced apoptosis of colon cancer stem cells (CSCs) [58;75], or through inhibition of NF-κB or COX-2 expression [61;68]. Research has shown that MSI confers cell resistance to apoptosis [76]. Consequently, microsatellite status might influence benefit from aspirin treatment. In vitro studies investigating long term aspirin exposure have already shown the selection for MSS and reduction of the MSI phenotype in colorectal and gastric cancer cell lines [77;78]. Goel *et al.* previously showed that aspirin treatment increased mismatch repair protein expression and apoptosis in CRC cells. Interestingly, growth inhibition of all human colon cancer cell lines was independent of microsatellite status, however, different growth regulatory mechanisms were responsible for this inhibition [79]. A recent study also confirmed that aspirin treatment induced NF-κB-driven apoptosis was independent of p53 expression and microsatellite status, suggesting that microsatellite status is not the predominant pathway responsible for aspirin anti-tumor activity [76]. In the preventive setting, for example in Lynch Syndrome families, aspirin could have an important influence on microsatellite status, thereby reducing MSI phenotype and thus cancer progression. However, since the MSI phenotype has been associated with improved survival [80], the survival benefit caused by aspirin will probably not be influenced by the microsatellite status of the primary tumor.

In summary, results from the above mentioned studies still keep us in the dark concerning aspirin's anti-cancer effects. Pooling of data from the different cohorts to improve statistical power in subgroup analyses followed by validation studies and randomized controlled trials are therefore eagerly awaited. In the Netherlands, a randomized placebo-controlled trial investigating low-dose aspirin (80 mg) after surgery in older colon cancer patients will start soon (Aspirin Trial, NTR 3370; EudraCT2011-004686-32). Possibly, more than one mechanism is responsible for the anti-cancer effects of aspirin. Different pathways should therefore be combined, also taken into account that the molecular mechanisms responsible for the anti-cancer effects of aspirin in the adjuvant setting may differ from the ones in the preventive setting.

PART THREE: PRECISION MEDICINE IN COLORECTAL CANCER AND FUTURE PERSPECTIVES

The TNM stage proved to fall short in clinical practice and needs to be supplemented with additional biomarkers to improve current staging and treatment allocation criteria substantially. A lot of research has been dedicated to the discovery and development of clinical prognostic and predictive biomarkers to improve diagnosis and to allocate optimal treatment modalities, introducing precision medicine in the multimodality treatment of cancer. By definition, precision medicine is a multi-faceted approach to medicine that integrates molecular and clinical research with patient data and clinical outcome, and places the patient at the center of all elements. Genomic, epigenomic, patient- and environmental data are studied together to understand individual disease patterns and to design preventive, diagnostic, and therapeutic solutions.

Unfortunately, in spite of a vast amount of available literature on biomarkers in CRC, only a few biomarkers are used on request in clinical practice nowadays, like *KRAS*, *BRAF*, MSI and the Onco*type* DX Colon Cancer Assay for determining whether to treat metastatic CRC patients with cetuximab or panitumumab, for the evaluation of Lynch syndrome and to inform treatment planning in stage II and III colon cancer patients.

In **Chapter 8** we have given an overview of a number of frequently studied biomarkers in CRC and emphasized on the difficulties and controversies that withhold clinical introduction of these biomarkers. In this review we have stated that there is insufficient evidence to introduce other biomarkers in clinical practice. Possible explanations are the use of divergent patient selection criteria, lack of consensus in performing studies and absence of validation studies.

Previously, a stepwise program for the introduction of biomarkers in clinical practice was developed with the first step being biomarker development in a preclinical, exploratory setting, subsequently followed by verification of this biomarker in a large retrospective study, validation and finally confirmation in a prospective randomized controlled trial [81]. Future studies should focus on following this program and standardized methods for performing studies, according to Good Clinical Practice recommendations, have to be developed. Furthermore, since tumor cells may acquire multiple capabilities during tumor development [15], the combination of biomarkers may provide greater prognostic and predictive value than the use of one single marker.

Over the last decade genomic profiling demonstrated its promising prognostic and predictive value in precision medicine and is therefore increasingly used in multidisciplinary consultations for risk-assessment and subsequent treatment planning of the individual cancer patient. The added value of genomic profiling for systemic therapy seems clear. In **Chapter 9** we have focused on the impact of genomic profiling on surgical decision-making. Apart from some single-gene mutations, genomic tumor profiling

in current clinical practice merely impacts surgical decision-making indirectly, as genomic tumor profiling of the biopsy might influence timing, extent and type of surgery by means of optimal tumor shrinkage through targeted neo-adjuvant therapy. Possibly, this may also lead to a wait-and-see approach in case of a pathological complete response (pCR). However, some issues should be resolved before genomic profiling has a clear influence on surgery, such as lack of clarity how to assess a pCR, the ideal timing of clinical, radiological and pathological assessment of response, the uncertainty of the long-term efficacy of this strategy, new follow-up protocols and the question of when to have surgery after neo-adjuvant treatment.

To achieve precision medicine in the future some important steps have to be taken. First, to increase clinical applicability, studies investigating biomarkers should focus on using standardized methods and comparable patient selection criteria in order to validate the results. Second, as current cancer research mainly focuses on the genotypical approach of cancer treatment, which is believed to alter cancer treatment radically in the near future, the phenotype of the cancer patient is ignored. In our greying society, cancer patients often suffer from one or more comorbid conditions, which should be

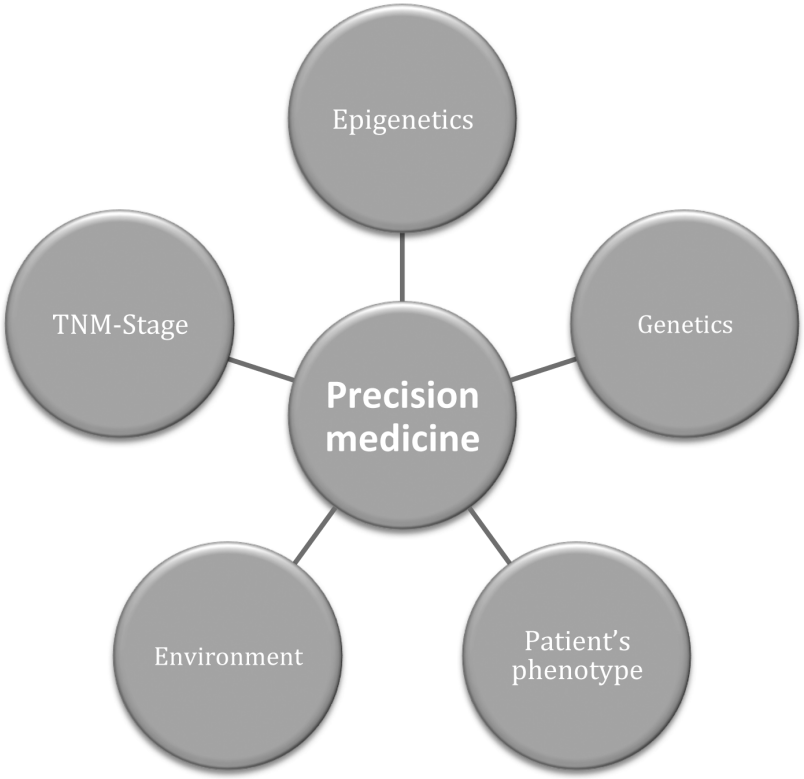


Figure 4: Precision medicine in the multimodality treatment of cancer.
By definition, precision medicine is a multi-faceted approach to medicine that integrates molecular and clinical research with patient data and outcomes and places the patient at the center of all elements.

taken into account when making cancer treatment decisions. Both a direct effect of comorbidity (competing risk of mortality) as well as the interaction with cancer must be weighed in these treatment decisions. Thus, parallel to the existing TNM stage for treatment allocation and the exciting new developments of the epigenetic and genetic fingerprint of the tumor, phenotypic profiling must be incorporated in the treatment approach of an individual patient. Finally, specialists involved in cancer management need to join forces and create a collaborative multidisciplinary approach to provide the most efficient and tolerated treatment in order to achieve precision medicine as ultimate goal (Figure 4).

REFERENCE LIST

(1) Kapiteijn E, Kranenbarg EK, Steup WH et al. Total mesorectal excision (TME) with or without preoperative radiotherapy in the treatment of primary rectal cancer. Prospective randomised trial with standard operative and histopathological techniques. Dutch ColoRectal Cancer Group. *Eur J Surg* 1999;165:410-420.

(2) Moertel CG, Fleming TR, Macdonald JS et al. Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. *N Engl J Med* 1990;322:352-358.

(3) Andre T, Boni C, Mounedji-Boudiaf L et al. Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. *N Engl J Med* 2004;350:2343-2351.

(4) Andre T, Boni C, Navarro M et al. Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIC trial. *J Clin Oncol* 2009;27:3109-3116.

(5) Andre T, Sargent D, Tabernero J et al. Current issues in adjuvant treatment of stage II colon cancer. *Ann Surg Oncol* 2006;13:887-898.

(6) Bosset JF, Collette L, Calais G et al. Chemotherapy with preoperative radiotherapy in rectal cancer. *N Engl J Med* 2006;355:1114-1123.

(7) Bujko K, Glynne-Jones R, Bujko M. Adjuvant chemotherapy for rectal cancer. *Ann Oncol* 2010;21:2443.

(8) Glimelius B, Dahl O, Cedermark B et al. Adjuvant chemotherapy in colorectal cancer: a joint analysis of randomised trials by the Nordic Gastrointestinal Tumour Adjuvant Therapy Group. *Acta Oncol* 2005;44:904-912.

(9) Peeters KC, van de Velde CJ, Leer JW et al. Late side effects of short-course preoperative radiotherapy combined with total mesorectal excision for rectal cancer: increased bowel dysfunction in irradiated patients--a Dutch colorectal cancer group study. *J Clin Oncol* 2005;23:6199-6206.

(10) Koebrugge B, Vogelaar FJ, Lips DJ et al. The number of high-risk factors is related to outcome in stage II colonic cancer patients. *Eur J Surg Oncol* 2011;37:964-970.

(11) Greene FL, Sobin LH. The staging of cancer: a retrospective and prospective appraisal. *CA Cancer J Clin* 2008;58:180-190.

(12) Gunderson LL, Sargent DJ, Tepper JE et al. Impact of T and N stage and treatment on survival and relapse in adjuvant rectal cancer: a pooled analysis. *J Clin Oncol* 2004;22:1785-1796.

(13) Kahlenberg MS, Sullivan JM, Witmer DD, Petrelli NJ. Molecular prognostics in colorectal cancer. *Surg Oncol* 2003;12:173-186.

(14) Kozak KR, Moody JS. The impact of T and N stage on long-term survival of rectal cancer patients in the community. *J Surg Oncol* 2008;98:161-166.

(15) Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57-70.

(16) Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 2011;331:1565-1570.

(17) Hokland M, Kuppen PJ. Natural killer cells: from "disturbing" background to central players of immune responses. *Mol Immunol* 2005;42:381-383.

(18) Menon AG, Morreau H, Tollenaar RA et al. Down-regulation of HLA-A expression correlates with a better prognosis in colorectal cancer patients. *Lab Invest* 2002;82:1725-1733.

(19) Watson NF, Ramage JM, Madjd Z et al. Immunosurveillance is active in colorectal cancer as down-regulation but not complete loss of MHC class I expression correlates with a poor prognosis. *Int J Cancer* 2006;118:6-10.

(20) Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nat Immunol* 2002;3:999-1005.

(21) Wischhusen J, Waschbisch A, Wiendl H. Immune-refractory cancers and their little helpers--an extended role for immunetolerogenic MHC molecules HLA-G and HLA-E? *Semin Cancer Biol* 2007;17:459-468.

(22) Liu F, Lang R, Zhao J et al. CD8(+) cytotoxic T cell and FOXP3(+) regulatory T cell infiltration in relation to breast cancer survival and molecular subtypes. *Breast Cancer Res Treat* 2011;130:645-655.

(23) Bukur J, Jasinski S, Seliger B. The role of classical and non-classical HLA class I antigens in human tumors. *Semin Cancer Biol* 2012;22:350-358.

(24) Lin A, Zhang X, Xu HH, Xu DP, Ruan YY, Yan WH. HLA-G expression is associated with metastasis and poor survival in the Balb/c nu/nu murine tumor model with ovarian cancer. *Int J Cancer* 2012;131:150-157.

(25) Popat S, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. *J Clin Oncol* 2005;23:609-618.

(26) Soreide K, Janssen EA, Soiland H, Korner H, Baak JP. Microsatellite instability in colorectal cancer. *Br J Surg* 2006;93:395-406.

(27) Dierssen JW, de Miranda NF, Ferrone S et al. HNPCC versus sporadic microsatellite-unstable colon cancers follow different routes toward loss of HLA class I expression. *BMC Cancer* 2007;7:33.

(28) Mouradov D, Domingo E, Gibbs P et al. Survival in stage II/III colorectal cancer is independently predicted by chromosomal and microsatellite instability, but not by specific driver mutations. *Am J Gastroenterol* 2013.

(29) Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 2012;487:330-337.

(30) Bedi A, Pasricha PJ, Akhtar AJ et al. Inhibition of apoptosis during development of colorectal cancer. *Cancer Res* 1995;55:1811-1816.

(31) Ramachandran A, Madesh M, Balasubramanian KA. Apoptosis in the intestinal epithelium: its relevance in normal and pathophysiological conditions. *J Gastroenterol Hepatol* 2000;15:109-120.

(32) Takano Y, Saegusa M, Ikenaga M, Mitomi H, Okayasu I. Apoptosis of colon cancer: comparison with Ki-67 proliferative activity and expression of p53. *J Cancer Res Clin Oncol* 1996;122:166-170.

(33) Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646-674.

(34) de Bruin EC, van de Velde CJ, van de Pas S et al. Prognostic value of apoptosis in rectal cancer patients of the dutch total mesorectal excision trial: radiotherapy is redundant in intrinsically high-apoptotic tumors. *Clin Cancer Res* 2006;12:6432-6436.

(35) de Heer P, de Bruin EC, Klein-Kranenbarg E et al. Caspase-3 activity predicts local recurrence in rectal cancer. *Clin Cancer Res* 2007;13:5810-5815.

(36) Fluge O, Gravdal K, Carlsen E et al. Expression of EZH2 and Ki-67 in colorectal cancer and associations with treatment response and prognosis. *Br J Cancer* 2009;101:1282-1289.

(37) Jonges LE, Nagelkerke JF, Ensink NG et al. Caspase-3 activity as a prognostic factor in colorectal carcinoma. *Lab Invest* 2001;81:681-688.

(38) Kimura T, Tanaka S, Haruma K et al. Clinical significance of MUC1 and E-cadherin expression, cellular proliferation, and angiogenesis at the deepest invasive portion of colorectal cancer. *Int J Oncol* 2000;16:55-64.

(39) Salminen E, Palmu S, Vahlberg T, Roberts PJ, Soderstrom KO. Increased proliferation activity measured by immunoreactive Ki67 is associated with survival improvement in rectal/recto sigmoid cancer. *World J Gastroenterol* 2005;11:3245-3249.

(40) Kim GP, Colangelo LH, Wieand HS et al. Prognostic and predictive roles of high-degree microsatellite instability in colon cancer: a National Cancer Institute-National Surgical Adjuvant Breast and Bowel Project Collaborative Study. *J Clin Oncol* 2007;25:767-772.

(41) Okon K, Demczuk S, Klimkowska A et al. Correlation of microsatellite status, proliferation, apoptotic and selected immunohistochemical markers in colorectal carcinoma studied with tissue microarray. *Pol J Pathol* 2006;57:105-111.

(42) Dolcetti R, Viel A, Doglioni C et al. High prevalence of activated intraepithelial cytotoxic T lymphocytes and increased neoplastic cell apoptosis in colorectal carcinomas with microsatellite instability. *Am J Pathol* 1999;154:1805-1813.

(43) O'Connell MJ, Lavery I, Yothers G et al. Relationship between tumor gene expression and recurrence in four independent studies of patients with stage II/III colon cancer treated with surgery alone or surgery plus adjuvant fluorouracil plus leucovorin. *J Clin Oncol* 2010;28:3937-3944.

(44) Gray RG, Quirke P, Handley K et al. Validation study of a quantitative multigene reverse transcriptase-polymerase chain reaction assay for assessment of recurrence risk in patients with stage II colon cancer. *J Clin Oncol* 2011;29:4611-4619.

(45) Ramsey SD, Barlow WE, Gonzalez-Angulo AM et al. Integrating comparative effectiveness design elements and endpoints into a phase III, randomized clinical trial (SWOG S1007) evaluating oncotypeDX-guided management for women with breast cancer involving lymph nodes. *Contemp Clin Trials* 2013;34:1-9.

(46) Sparano JA, Paik S. Development of the 21-gene assay and its application in clinical practice and clinical trials. *J Clin Oncol* 2008;26:721-728.

(47) Burn J, Gerdes AM, Macrae F et al. Long-term effect of aspirin on cancer risk in carriers of hereditary colorectal cancer: an analysis from the CAPP2 randomised controlled trial. *Lancet* 2011;378:2081-2087.

(48) Rothwell PM, Wilson M, Elwin CE et al. Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomised trials. *Lancet* 2010;376:1741-1750.

(49) Rothwell PM, Price JF, Fowkes FG et al. Short-term effects of daily aspirin on cancer incidence, mortality, and non-vascular death: analysis of the time course of risks and benefits in 51 randomised controlled trials. *Lancet* 2012.

(50) Bastiaannet E, Sampieri K, Dekkers OM et al. Use of aspirin postdiagnosis improves survival for colon cancer patients. *Br J Cancer* 2012;106:1564-1570.

(51) Chan AT, Ogino S, Fuchs CS. Aspirin use and survival after diagnosis of colorectal cancer. *JAMA* 2009;302:649-658.

(52) McCowan C, Munro AJ, Donnan PT, Steele RJ. Use of aspirin post-diagnosis in a cohort of patients with colorectal cancer and its association with all-cause and colorectal cancer specific mortality. *Eur J Cancer* 2013;49:1049-1057.

(53) Brown JR, DuBois RN. COX-2: a molecular target for colorectal cancer prevention. *J Clin Oncol* 2005;23:2840-2855.

(54) Chen WS, Wei SJ, Liu JM, Hsiao M, Kou-Lin J, Yang WK. Tumor invasiveness and liver metastasis of colon cancer cells correlated with cyclooxygenase-2 (COX-2) expression and inhibited by a COX-2-selective inhibitor, etodolac. *Int J Cancer* 2001;91:894-899.

(55) Wang D, DuBois RN. The role of COX-2 in intestinal inflammation and colorectal cancer. *Oncogene* 2010;29:781-788.

(56) Ofosu FA. Appropriate Assessment of the Functional Consequences of Platelet Cyclooxygenase-1 Inhibition by Aspirin in vivo. *Thromb Res* 2013.

(57) Midgley RS, McConkey CC, Johnstone EC et al. Phase III randomized trial assessing rofecoxib in the adjuvant setting of colorectal cancer: final results of the VICTOR trial. *J Clin Oncol* 2010;28:4575-4580.

(58) Cianferoni A, Schroeder JT, Kim J et al. Selective inhibition of interleukin-4 gene expression in human T cells by aspirin. *Blood* 2001;97:1742-1749.

(59) Dihlmann S, Siermann A, von Knebel DM. The nonsteroidal anti-inflammatory drugs aspirin and indomethacin attenuate beta-catenin/TCF-4 signaling. *Oncogene* 2001;20:645-653.

(60) Dihlmann S, Klein S, Doeberitz MM. Reduction of beta-catenin/T-cell transcription factor signaling by aspirin and indomethacin is caused by an increased stabilization of phosphorylated beta-catenin. *Mol Cancer Ther* 2003;2:509-516.

(61) Langley RE, Burdett S, Tierney JF, Cafferty F, Parmar MK, Venning G. Aspirin and cancer: has aspirin been overlooked as an adjuvant therapy? *Br J Cancer* 2011;105:1107-1113.

(62) Wang Y, Chen X, Zhu W, Zhang H, Hu S, Cong X. Growth inhibition of mesenchymal stem cells by aspirin: involvement of the WNT/beta-catenin signal pathway. *Clin Exp Pharmacol Physiol* 2006;33:696-701.

(63) Liao X, Lochhead P, Nishihara R et al. Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. *N Engl J Med* 2012;367:1596-1606.

(64) Samuels Y, Wang Z, Bardelli A et al. High frequency of mutations of the PIK3CA gene in human cancers. *Science* 2004;304:554.

(65) Barault L, Veyrie N, Jooste V et al. Mutations in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in a population-based series of colon cancers. *Int J Cancer* 2008;122:2255-2259.

(66) Liao X, Morikawa T, Lochhead P et al. Prognostic role of PIK3CA mutation in colorectal cancer: cohort study and literature review. *Clin Cancer Res* 2012;18:2257-2268.

(67) Whitehall VL, Rickman C, Bond CE et al. Oncogenic PIK3CA mutations in colorectal cancers and polyps. *Int J Cancer* 2012;131:813-820.

(68) Kaur J, Sanyal SN. PI3-kinase/Wnt association mediates COX-2/PGE(2) pathway to inhibit apoptosis in early stages of colon carcinogenesis: chemoprevention by diclofenac. *Tumour Biol* 2010;31:623-631.

(69) Uddin S, Ahmed M, Hussain A et al. Cyclooxygenase-2 inhibition inhibits PI3K/AKT kinase activity in epithelial ovarian cancer. *Int J Cancer* 2010;126:382-394.

(70) Labelle M, Begum S, Hynes RO. Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. *Cancer Cell* 2011;20:576-590.

(71) Reymond N, d'Agua BB, Ridley AJ. Crossing the endothelial barrier during metastasis. *Nat Rev Cancer* 2013;13:858-870.

(72) Placke T, Orgel M, Schaller M et al. Platelet-derived MHC class I confers a pseudonormal phenotype to cancer cells that subverts the antitumor reactivity of natural killer immune cells. *Cancer Res* 2012;72:440-448.

(73) Domingo E, Church DN, Sieber O et al. Evaluation of PIK3CA Mutation As a Predictor of Benefit From Nonsteroidal Anti-Inflammatory Drug Therapy in Colorectal Cancer. *J Clin Oncol* 2013.

(74) Bruno A, Dovizio M, Tacconelli S, Patrignani P. Mechanisms of the antitumoural effects of aspirin in the gastrointestinal tract. *Best Pract Res Clin Gastroenterol* 2012;26:e1-e13.

(75) Todaro M, Alea MP, Di Stefano AB et al. Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. *Cell Stem Cell* 2007;1:389-402.

(76) Din FV, Stark LA, Dunlop MG. Aspirin-induced nuclear translocation of NFkappaB and apoptosis in colorectal cancer is independent of p53 status and DNA mismatch repair proficiency. *Br J Cancer* 2005;92:1137-1143.

(77) Ruschoff J, Wallinger S, Dietmaier W et al. Aspirin suppresses the mutator phenotype associated with hereditary nonpolyposis colorectal cancer by genetic selection. *Proc Natl Acad Sci U S A* 1998;95:11301-11306.

(78) Yamamoto H, Itoh F, Fukushima H, Hinoda Y, Imai K. Overexpression of cyclooxygenase-2 protein is less frequent in gastric cancers with microsatellite instability. *Int J Cancer* 1999;84:400-403.

(79) Goel A, Chang DK, Ricciardiello L, Gasche C, Boland CR. A novel mechanism for aspirin-mediated growth inhibition of human colon cancer cells. *Clin Cancer Res* 2003;9:383-390.

(80) Bertagnolli MM, Redston M, Compton CC et al. Microsatellite instability and loss of heterozygosity at chromosomal location 18q: prospective evaluation of biomarkers for stages II and III colon cancer--a study of CALGB 9581 and 89803. *J Clin Oncol* 2011;29:3153-3162.

(81) Pepe MS, Etzioni R, Feng Z et al. Phases of biomarker development for early detection of cancer. *J Natl Cancer Inst* 2001;93:1054-1061.

CHAPTER 11

Nederlandse Samenvatting

Het carcinoom van de dikke darm en endeldarm (colorectaal carcinoom, CRC) staat op de derde plaats van meest voorkomende tumoren wereldwijd en is tevens een van de grootste veroorzakers van kanker-gerelateerde sterfte [1;2]. Ongeveer 20-25% van de patienten presenteren zich al met een gemetastaseerde ziekte tijdens diagnose en 20-25% van de patiënten ontwikkelen metastasen tijdens de progressie van deze ziekte, wat resulteert in een mortaliteit van 40-45% [3;4].

CRC ontstaat meestal spontaan, in 5% van de gevallen is het erfelijk, via een stapsgewijs proces van maligne ontaarding van gezonde cellen tot carcinoom, ook wel de adenoma-carcinoma sequentie genoemd [5]. Een aantal jaar geleden werd duidelijk dat tumorcellen voor deze maligne ontaarding zes biologische eigenschappen moeten verkrijgen, de zogenaamde 'hallmarks of cancer'[6]. Deze eigenschappen zijn autonomie van groeisignalen, ongevoeligheid voor groei-remmende signalen, invasieve groei en metastasering, immortaliteit, aanhoudende vaatnieuwvorming en ongevoeligheid voor apoptose (geprogrammeerde celdood). Recentelijk werden nog 2 belangrijke aanvullende eigenschappen erkend; reprogrammeren van het energiemetabolisme en ontsnappen aan herkenning door het immuunsysteem [7]. Tevens werd de rol van het micromilieu rondom de tumor, die verantwoordelijk is voor het verkrijgen van deze eigenschappen, erkend. Genetische instabiliteit in een cel is verantwoordelijk voor het verkrijgen van deze tumoreigenschappen.

De belangrijkste pijler van behandeling van CRC is chirurgische verwijdering van de tumor met meenemen van bijbehorende lymfeklieren. De laatste decennia heeft de behandeling van colorectaal carcinoom zich sterk ontwikkeld. Mede door de introductie van nieuwe operatietechnieken, zoals de Totale Mesorectale Excisie (TME) bij rectumcarcinoom, preoperatieve radiotherapie bij rectumcarcinoom en adjuvante chemotherapie is de overleving van patiënten met CRC sterk verbeterd. Recent zijn er ook aanwijzingen gevonden dat behandeling met aspirine een remmende werking op tumorvorming en een positieve invloed op de overleving van patiënten met CRC heeft [8-15].

Tegenwoordig wordt de keuze voor een bepaalde operatie en aanvullende therapie sterk beïnvloed door tumorstadiëring en locatie van de tumor. Echter, ongeveer 20-25% van de patiënten, die door een lage tumorstadiëring geen aanvullende therapie hebben gekregen, krijgen toch terugkeer van de ziekte binnen vijf jaar na diagnose [16]. Tevens kunnen de nieuwe aanvullende behandelingen voor aanzienlijke bijwerkingen zorgen en weten we uit onderzoek dat niet iedere patiënt baat heeft bij een bepaalde aanvullende therapie. Deze tekortkomingen in het huidige gebruik van tumorstadiëring kunnen resulteren in zowel onderbehandeling als overbehandeling van patiënten. Tumorstadiëring is daarom niet een optimaal beslismodel om de prognose van een patiënt te voorspellen en patiënten te selecteren voor aanvullende therapie. Huidige inzichten laten zien dat de onderliggende biologie van CRC ook invloed heeft op de prognose van de patiënt en het slagen van een aanvullende behandeling. Op basis van

zogenaamde biomarkers, die een weergave vormen van de onderliggende biologie en daarmee de kans op tumorgroei en metastasering reflecteren, kan een accurate voorspelling worden gedaan over de prognose van de patiënt (prognostische biomarkers) of het slagen van een behandeling (predictieve biomarkers). De groei van een tumor zou optimaal voorspeld kunnen worden door een combinatie van klinische, pathologische en biologische tumorkarakteristieken, resulterend in een optimale gepersonaliseerde behandeling.

Dit proefschrift richt zich op sleutelbiomarkers vanuit de moleculaire biologie van CRC, waarmee een voorspelling kan worden gedaan over de prognose van een patiënt en het slagen van een behandeling.

DEEL EEN: PROGNOSTISCHE BIOMARKERS IN COLORECTAAL CARCINOOM

Met behulp van prognostische biomarkers, die betrokken zijn bij maligne ontaarding van darmepitheelcellen en progressie van tumoren, kan een voorspelling worden gedaan over de prognose van een patiënt na resectie van de primaire tumor. Om dit te onderzoeken hebben we enkele biomarkers geselecteerd die behoren tot de 'hallmarks of cancer', zoals hierboven beschreven.

In **hoofdstuk 2 en 3** hebben we de prognostische waarde van belangrijke biomarkers, die gerelateerd zijn aan het immuunsysteem, in zowel coloncarcinoom als rectumcarcinoom onderzocht. Door middel van immunohistochemische kleuringen werd de tumorexpressie van Humaan Leukocyt Antigeen (HLA) klasse I, HLA-E en HLA-G en tumorinfiltratie van immunosuppressieve regulatoire T-cellen (Tregs) in kaart gebracht, met als doel het ontrafelen van ontsnappingsmechanismen van de tumor aan herkenning door het immuunsysteem. Er zijn aanwijzingen dat door genetische instabiliteit varianten van tumorcellen ontstaan, die een grotere kans hebben om aan het immuunsysteem te ontsnappen ('immunoediting'). Deze tumorcellen kunnen vervolgens verder uitgroeien en metastaseren [17-20] . Een aantal mechanismen zijn daarvoor verantwoordelijk. HLA klasse I moleculen presenteren antigenen aan cytotoxische T-lymphocyten (CTL), die hierdoor schadelijke cellen voor het lichaam, zoals tumorcellen, kunnen herkennen en opruimen. Verminderde expressie van HLA klasse I op tumorcellen kan er dus voor zorgen dat deze cellen niet meer worden herkend en ontsnappen aan vernietiging door CTL [21]. Tumorcellen kunnen naast het verminderen van de HLA klasse I expressie op hun celmembraan HLA-G tot expressie brengen. HLA-G komt zelden voor in gezonde weefsels, maar vertoont wel expressie in tumoren [22]. HLA-E is daarentegen wel aanwezig op verscheidene gezonde weefsels en correleert met expressie van HLA klasse I [23]. Onderzoek heeft aangetoond dat tumorexpressie van HLA-E en HLA-G ervoor zorgt dat natural-killer (NK) cellen niet geactiveerd worden, waardoor tumorcellen nog verder

kunnen ontsnappen aan het immuunsysteem [23-25]. Tenslotte kan infiltratie met Tregs in het micromilieu rondom de tumor zorgen voor remming van de activiteit van CTL [26;27].

In 285 'stadium I-IV' patiënten met coloncarcinoom **(hoofdstuk 2)** was totale tumorafschakeling van HLA klasse I gerelateerd aan een betere overleving. Deze bevinding zou kunnen worden verklaard doordat deze tumorcellen, zodra ze metastaseren naar de bloedbaan, opgeruimd worden door NK-cellen [28;29]. Wanneer we de immuunmarkers combineerden in de statistische analyse zagen we 3 verschillende overlevingsgroepen ontstaan, die van significant prognostische waarde waren. Totale afschakeling van HLA klasse I in combinatie met afwezigheid van HLA-E en HLA-G op de tumor was geassocieerd met de beste overleving. Door afwezigheid van HLA-E en HLA-G op de tumorcel werden deze tumoren waarschijnlijk nog beter opgeruimd door NK-cellen [24;25]. Patiënten met tumoren met verminderde expressie van HLA klasse I en weinig infiltratie van Tregs waren geassocieerd met de slechtste overleving. Deze tumoren zijn waarschijnlijk door activiteit van CTL veranderd in een tumorvariant met verminderde expressie van HLA klasse I, zodat ze verder kunnen ontsnappen aan herkenning door het immuunsysteem. Hierdoor kunnen deze tumorcellen nu niet goed meer door CTL opgeruimd worden, resulterend in verdere groei of metastasering van de tumorcellen. De afwezigheid van Tregs in deze tumoren versterkt onze hypothese aangezien CTL en Tregs tegengestelde werkingsmechanismen vertonen [30]. In deze tumoren met een slechte prognose zijn Tregs waarschijnlijk niet noodzakelijk, omdat de aanwezigheid van CTL activiteit schaars is. Doordat er nog partiële expressie van HLA klasse I aanwezig is kunnen deze tumoren mogelijk ook niet goed opgeruimd worden door NK-cellen.

In **hoofdstuk 3** hebben we de prognostische waarde van dezelfde biomarkers, wederom onafhankelijk van elkaar en gecombineerd, in 495 rectumtumoren van de Nederlandse TME-studie onderzocht. Zowel expressie van HLA klasse I als een hoge Treg infiltratie was geassocieerd met een betere overleving. Interessant was dat ook een sterke HLA-G expressie geassocieerd was met een betere overleving. Dit resultaat is opmerkelijk, aangezien expressie van HLA-G als secundair ontsnappingsmechanisme NK-cellen kan remmen om tumorcellen met verminderde expressie of totale afschakeling van HLA klasse I op te ruimen [24;25]. De reden hiervoor is onduidelijk. Immuun-regulatie in tumorcellen blijft een complex fenomeen, waarvan nog niet alle immuun-gerelateerde ontsnappingsmechanismen in kaart gebracht zijn. Mogelijk speelt HLA-G geen grote rol in rectumcarcinoom. Wanneer de immuunmarkers werden gecombineerd in de statistische analyse, zagen we wederom 3 overlevingsgroepen ontstaan. De prognose van de patiënt nam toe bij toename van het aantal markers die waren gerelateerd aan een goede prognose, zoals expressie van HLA klasse I, hoge Treg infiltratie en expressie van HLA-G. Interessant was dat patiënten met tumoren met totale afschakeling van HLA klasse I, expressie van HLA-G en weinig infiltratie met Tregs de slechtste prognose hadden. Deze tumoren hebben zich mogelijk zodanig aangepast dat ze goed kunnen

ontsnappen aan het immuunsysteem. Door verlies van HLA-expressie is er geen herkenning door CTL en door expressie van HLA-G ook geen opruiming van de tumorcellen door NK-cellen. HLA-G lijkt in deze fase van 'immunoediting', wanneer HLA klasse I totaal afgeschakeld is, wel een belangrijke rol in de overleving van tumorcellen te spelen.

De uitgevoerde studies tonen aan dat er waarschijnlijk verschillen bestaan tussen coloncarcinoom en rectumcarcinoom. De meeste studies die onderzoek hebben gedaan naar de relatie tussen immuunmarkers en prognose hebben deze studies uitgevoerd in patiënten-cohorten met zowel rectumtumoren als colontumoren [31;32]. Deze studies laten vaak zien dat totale afschakeling geassocieerd is met een betere overleving. Echter, in rectumcarcinoom was HLA-expressie gerelateerd aan een betere overleving. Mogelijk zijn biologische verschillen tussen rectum en colon verantwoordelijk voor deze verschillen. Een van de biologische verschillen tussen rectum- en colontumoren is het fenomeen microsatellietinstabiliteit (MSI). Ongeveer 50% van de proximale coloncarcinomen vertonen MSI, terwijl bijna alle tumoren in het rectum juist microsatellietstabiele (MSS) tumoren zijn [33;34]. MSI is geassocieerd met totale afschakeling van HLA klasse I en een betere overleving, wat de resultaten kan vertroebelen [35;36]. Daarom is het noodzaak om, wanneer naar HLA klasse I expressie gekeken wordt in colorectale tumoren, de microsatellietstatus van de tumor in acht te nemen. Helaas was het aantal patiënten met MSI-tumoren in ons coloncarcinoomcohort te klein om separate analyses in MSI- en MSS-tumoren uit te voeren.

In **hoofdstuk 4** hebben we de prognostische waarde van biomarkers die gerelateerd zijn aan apoptose en proliferatie onderzocht. Een disbalans tussen deze twee processen kan bijdragen aan het ontstaan en onderhouden van tumorgroei en –ontwikkeling [6;7]. Verschillende studies laten tegenstrijdige resultaten zien wanneer gekeken wordt naar de prognostische waarde van apoptose of proliferatie, met name tussen tumoren afkomstig van het colon of rectum [37-40]. Ook lijkt er sprake te zijn dat de microsatellietstatus van invloed kan zijn op deze processen [39;41;42]. In 285 'stadium I-IV' patiënten met coloncarcinoom hebben we de mate van apoptose en proliferatie onderzocht door middel van immunohistochemische kleuringen met antilichamen tegen respectievelijk actief caspase-3 (een apoptose-inducerend enzym) en ki67 (proliferatiemarker). Deze studie liet zien dat patiënten met tumoren met zowel een sterke tumorcelproliferatie als apoptose de beste overleving hebben. Interessant was echter dat de uitkomst van deze studie varieerde met de locatie van de tumor en waarschijnlijk met de microsatellietstatus van de tumor. Helaas was het aantal MSI-tumoren in ons cohort te klein om separate analyses uit te voeren. Echter, MSI werd wel significant meer waargenomen in rechtszijdige tumoren. Een balans tussen apoptose en proliferatie (aanwezigheid van een sterke tumorcelproliferatie en aanwezigheid van apoptose, of afwezigheid van beide processen) was geassocieerd met een betere overleving in linkszijdige colontumoren. Zoals je kan verwachten van sterk prolifererende tumoren, hadden patiënten met

linkszijdige tumoren waarin sprake was van een sterke proliferatie en afwezigheid van apoptose de slechtste overleving. In de rechtszijdige tumoren daarentegen, lieten de tumoren met een sterke proliferatie en afwezigheid van apoptose een betere overleving zien. Locatie van de tumor of tumormicrosatellietstatus hebben dus mogelijk invloed op de prognostische waarde van deze markers gezamenlijk. Eerder onderzoek heeft laten zien dat MSI-tumoren vaak veel proliferatie laten zien. Tevens vertonen MSI tumoren veel genmutaties, waardoor sprake is van een verhoogde productie van abnormale eiwitten [41;42]. Dit leidt mogelijk tot een sterke immuunreactie met als gevolg toename van apoptose in de tumoren. De betere prognose van deze patiënten met rechtszijdige tumoren met een sterke proliferatie zou hierdoor kunnen worden verklaard [43]. In de toekomst zullen deze resultaten gevalideerd moeten worden, waarbij rekening moet worden gehouden met de locatie en de microsatellietstatus van de tumor.

In **hoofdstuk 5** hebben we een validatie uitgevoerd van de 12-gene Colon Cancer Recurrence Score® Assay in 297 patiënten met rectumcarcinoom uit de Nederlandse TME-trial die een operatieve verwijdering van hun tumor hebben ondergaan, zonder preoperatieve bestraling. Deze test, die de expressie meet van 12 genen, was eerder al gevalideerd in meerdere trials in patiënten met stadium II coloncarcinoom [44;45]. Gebaseerd op de expressie van de genen worden patiënten ingedeeld in 3 groepen; een laag risico, gemiddeld risico en hoog risico op terugkeer van de ziekte. In deze validatiestudie in rectumcarcinoom voorspelde de Recurrence Score® (RS) het risico op terugkeer van de ziekte, het risico op afstandsmetastasen en rectumcarcinoom-specifieke overleving. Het effect was het meest zichtbaar in stadium II rectumcarcinoom. RS zou in de toekomst mogelijk in de kliniek gebruikt kunnen worden om patiënten met een hoog of laag risico op terugkeer van de ziekte te selecteren voor respectievelijk wel of geen adjuvante chemotherapie.

DEEL TWEE: BEHANDELING VAN COLONCARCINOOM EN PREDICTIEVE BIOMARKERS

Onderzoek toont aan dat aspirinegebruik na de diagnose de overleving en mortaliteit van patiënten met CRC sterk verbetert [9-11;46]. In **hoofdstuk 6** hebben we het effect van aspirinegebruik (80 mg) na de diagnose op de overleving onderzocht door een subanalyse te verrichten in ouderen (≥ 70 jaar) met coloncarcinoom uit het cohort dat eerder is gebruikt door Bastiaannet *et al.*[46]. In deze studie was aspirinegebruik na de diagnose significant geassocieerd met een sterk verbeterde overleving in ouderen met coloncarcinoom vergeleken met niet-gebruikers. Al deze studies impliceren dat het goed getolereerde en goedkope aspirine mogelijk gebruikt kan worden als een nieuwe

adjuvante therapie, wat zeker bij ouderen met colon carcinoom een grote klinische doorbraak zal zijn.

Het exacte mechanisme achter dit fenomeen is nog niet bekend. Mogelijk spelen bepaalde enzymen en genen daarbij een rol, waaronder het enzym COX-2 (cyclooxygenase-2), COX-1 (cyclooxygenase-1) en het gen *PIK3CA*. COX-1 is verantwoordelijk voor trombocytenaggregatie door productie van TXA2 in trombocyten, waarop aspirine aangrijpt [47]. COX-2 is een enzym dat betrokken is bij de prostaglandine productie en maligne ontaarding van epitheliale cellen, wat kan worden geremd door aspirine [48;49]. Ongeveer 70% van de CRCs vertonen expressie van COX-2 [9] . Recent onderzoek liet echter ook zien dat aspirine mogelijk tumorgroei remt en apoptose induceert door het blokkeren van de phosphatidylinositol 3-kinase (*PIK3CA*) signaleringroute, die verantwoordelijk is voor de aansturing van COX-2 [50]. Deze route speelt een belangrijke rol in tumorgroei en progressie [51]. In 15-20% van de CRCs worden mutaties in het *PIK3CA* gevonden. Aspirine zou mogelijk alleen een positieve invloed hebben op de overleving van patiënten met een CRC indien er sprake is van een *PIK3CA*-mutatie in de tumor [52] of verhoogde expressie van COX-2 [9].

Omdat aspirinegebruik gepaard kan gaan met negatieve bijwerkingen bij de patiënt en mogelijk kan resulteren in overbehandeling, hebben we in **hoofdstuk 7** onderzoek gedaan naar predictieve biomarkers die kunnen voorspellen welke patiënten met coloncarcinoom baat hebben bij aspirinetherapie na de diagnose. In de bloedbaan kan het metastaseringsvermogen van tumorcellen worden beïnvloed door omgevingsfactoren, waaronder trombocyten en cellen afkomstig van het beenmerg [53]. Zodra tumorcellen de bloedbaan binnen dringen, komen ze in contact met trombocyten [54]. Er wordt gedacht dat trombocyten metastaserende tumorcellen in de bloedbaan beschermen tegen immuuncellen, zoals NK-cellen. Doordat trombocyten als het ware een schild vormen rondom tumorcellen wordt de NK-reactiviteit beïnvloed [55]. De interactie tussen tumorcellen en trombocyten resulteert in verplaatsing van HLA klasse I expressie van de trombocyt naar het celmembraan van de tumorcel, resulterend in een HLA klasse I fenotype. Hierdoor wordt NK-cel activiteit geblokkeerd. Doordat HLA klasse I afkomstig is van de trombocyt, wat niet als lichaamsvreemd herkend wordt, worden CTL ook niet geactiveerd [55].

Aspirine remt de trombocytenaggregatie [47] en aannemelijk is dat het schild van trombocyten rondom de tumorcel ook geremd wordt, met als gevolg dat trombocyten de metastaserende tumorcellen niet meer kunnen beschermen tegen afbraak door immuuncellen, zoals de NK-cellen. NK-cellen herkennen en elimineren bij voorkeur cellen met totale afschakeling of verminderde expressie van HLA klasse I [55]. De hypothese van deze studie was dan ook dat de overlevingswinst van aspirinegebruik na de diagnose coloncarcinoom geassocieerd is met tumoren met totale afschakeling of verminderde expressie van HLA klasse I. Echter, in strijd met onze hypothese, vonden we in deze

studie, waarbij tumorweefsel van 999 patiënten met coloncarcinoom beschikbaar was, dat aspirinegebruik na de diagnose alleen een positieve invloed heeft op overleving indien er sprake is van HLA expressie op de tumor. Er zijn twee mogelijke verklaringen voor dit fenomeen. Ten eerste zou aspirinegebruik kunnen zorgen voor verstoring van trombocyten, waardoor tumorcellen met HLA klasse I nu meer toegankelijk worden voor CTL activiteit. Ten tweede zorgt direct contact tussen trombocyten en tumorcellen voor secretie van TGF-β en activatie van de NF-κB signaleringsroute, wat resulteert in een epitheliaal-mesenchymaal fenotype met een verhoogd metastaseringsvermogen van de circulerende tumorcellen [53]. Aspirine zou de NF-κB signalering tussen trombocyten en tumorcellen, die mogelijk afhankelijk is van een intacte HLA klasse I expressie, kunnen remmen. Hierdoor kan de epitheliale-mesenchymale transitie in circulerende tumorcellen worden voorkomen met als gevolg een vermindering van het metastaseringsvermogen.

Onze studie heeft niet kunnen bevestigen dat aspirinegebruik alleen een positief effect heeft op de overleving van patiënten met coloncarcinoom wanneer er sprake is van verhoogde COX-2 expressie of een *PIK3CA* mutatie. Een farmacologische oorzaak kan daaraan ten grondslag liggen. Farmacologische studies naar het gebruik van aspirine tonen aan dat een lage dosis (80 mg), die gebruikt is in onze studie, niet genoeg is om permanent COX-2 te acetyleren, maar wel de optimale dosis is voor trombocyten remming [56]. In de studies die hebben aangetoond dat *PIK3CA* en COX-2 expressie predictieve biomarkers zijn voor de positieve invloed van aspirinegebruik in CRC was de dosis 325 mg [57;58].

Mogelijk heeft aspirine meerdere werkingsmechanismen in CRC; een direct effect op trombocyten door remming van COX-1, nodig voor het remmen van metastasering en waarvoor een lage dosering aspirine genoeg is; en een tweede mechanisme dat in de weefsels COX-2 expressie remt, waarvoor een hogere en meer frequente dosering nodig is.

DEEL DRIE: GEPERSONALISEERDE BEHANDELING VAN COLORECTAAL CARCINOOM

De huidige classificering middels de TNM stadiëring, waarbij rekening wordt gehouden met de tumor zelf (T), de betrokken lymfeklieren (N) en metastasering op afstand (M), blijkt niet een optimaal handvat te zijn voor artsen om een bepaalde behandelingsstrategie te bepalen. Het is daarom belangrijk om additionele biomarkers te vinden die de huidige tumorstadiëring kunnen verbeteren. Met gebruik van biomarkers naast de bestaande tumorstadiëring zou de prognose van een patiënt beter kunnen worden ingeschat en de behandeling worden geoptimaliseerd. Het uiteindelijke doel van de ontdekking en ontwikkeling van al deze klinische prognostische en predictieve

biomarkers is een optimaal multidisciplinair bepaalde behandeling gericht op de individuele patiënt. Deze gepersonaliseerde behandeling integreert moleculaire en klinische tumoreigenschappen met patiëntkarakteristieken. (Epi)genetische eigenschappen en externe factoren worden samen met patiëntkarakteristieken bestudeerd, waardoor de ziekteprogressie van een individuele patiënt beter in kaart kan worden gebracht en betere preventieve, diagnostische en therapeutische methoden kunnen worden ontwikkeld.

Helaas worden, ondanks een enorme overvloed aan gepubliceerd onderzoek naar biomarkers, in de praktijk op aanvraag slechts enkele biomarkers gebruikt. Voorbeelden hiervan zijn *KRAS* en *BRAF* om te bepalen of een patiënt met gemetastaseerd CRC geschikt is voor behandeling met cetuximab of panitumumab, MSI voor het bepalen van het erfelijke Lynch syndroom en de Onco*type* DX Colon Cancer Assay om te bepalen of aanvullende behandeling in stadium II en III coloncarcinoom wenselijk kan zijn. In **hoofdstuk 8** hebben we een overzicht gemaakt van de meest bestudeerde biomarkers in CRC. Echter, de meeste biomarkers worden, ondanks de mooie resultaten die zijn gepubliceerd, niet gebruikt in de kliniek. Het gebrek aan consensus in de patiënten selectiecriteria en het uitvoeren van studies, en de afwezigheid van validatie studies zijn mogelijke oorzaken die hieraan ten grondslag liggen. De moeilijkheden en controverses die gepaard gaan met de klinische introductie van een biomarker worden verder in dit hoofdstuk besproken. Uit dit overzicht hebben we moeten concluderen dat er op dit moment onvoldoende bewijs is om naast *KRAS*, *BRAF*, MSI en Onco*type* DX andere biomarkers in de kliniek te gebruiken.

In de toekomst zou een stappenplan voor de klinische introductie van een biomarker kunnen worden gebruikt om dit probleem op te lossen [59]. De eerste stap van dit programma zou bestaan uit ontwikkeling van een biomarker in een preklinische exploratieve setting, gevolgd door verificatie van de biomarker in een grote retrospectieve studie, validatie en uiteindelijk bevestiging van de waarde van de biomarker in een prospectieve gerandomiseerde trial. Tevens zou een combinatie van biomarkers meer prognostische en predictieve waarde kunnen bieden dan het gebruik van één enkele biomarker.

De laatste jaren heeft genotypering ook een veelbelovende voet aan de grond gekregen in de behandeling van CRC, waarbij met name de risicopredictie en de behandelingsstrategie van de individuele patiënt centraal staan. Alhoewel de toegevoegde waarde van genotypering voor het bepalen van systemische therapie duidelijk lijkt, zoals ook beschreven in hoofdstuk 5, is de impact van genotypering op chirurgisch vlak onduidelijk. In **hoofdstuk 9** wordt de rol van genotypering in de chirurgische besluitvorming bediscussieerd. Behoudens enkele afzonderlijke genetische mutaties die een directe invloed hebben op chirurgisch ingrijpen, zoals *BRCA* mutaties in borstkanker waarvoor een profylactische bilaterale mastectomie wordt geadviseerd, is er tot op he-

den geen directe relatie tussen genotypering en chirurgische besluitvorming. Indirect kan genotypering echter wel invloed hebben op de chirurgische besluitvorming. Genotypering van een preoperatief biopt kan leiden tot een gerichte neo-adjuvante therapie, leidend tot een mogelijke tumorregressie, met als gevolg dat de timing en omvang van de operatie worden beïnvloed. Indien door een gerichte neo-adjuvante therapie complete remissie van de tumor optreedt, zou zelfs een 'wait-and-see' benadering tot de mogelijkheden behoren. Echter, voordat genotypering een duidelijk rol zal spelen in de chirurgische besluitvorming zal er eerst meer duidelijkheid moeten zijn over hoe een complete remissie wordt vastgesteld, hoeveel tijd er tussen de gegeven gerichte therapie en het vaststellen van het therapie effect moet zitten, wat de lange termijn effecten van deze strategie zijn en wanneer de operatie na deze gerichte neo-adjuvante therapie moet plaatsvinden.

Om uiteindelijk de veelbelovende gepersonaliseerde behandeling met behulp van biomarkers en genotypering van kankerpatiënten te bereiken, moeten belangrijke stappen worden genomen. Ten eerste is het, zoals hierboven beschreven, voor de ontwikkeling en validatie van biomarkers en genotyperingsprofielen belangrijk om gestandaardiseerde methoden en vergelijkbare patiëntcohorten te gebruiken, waarmee de integratie in de kliniek verbeterd kan worden. Ten tweede is het in onze vergrijzende populatie ook niet onbelangrijk om het fenotype van een patiënt in acht te nemen. Zowel de comorbiditeiten van oudere patiënten als de invloed van deze comorbiditeiten op de tumor moeten worden meegenomen in een gewogen patiëntgerichte behandelingsstrategie. Tenslotte is het van groot belang dat alle specialisten die betrokken zijn bij kankerbehandeling hun krachten nog verder bundelen, waardoor de reeds bestaande multidisciplinaire behandeling van tumoren verder uitgebreid kan worden met kennis op het gebied van tumorbiologie. Door inachtneming van deze belangrijke factoren zal in de toekomst aan iedere individuele patiënt de meest efficiënte en draaglijke behandeling kunnen worden geboden.

REFERENCE LIST

(1) Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010;127:2893-2917.

(2) Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011;61:69-90.

(3) La Vecchia C., Bosetti C, Lucchini F et al. Cancer mortality in Europe, 2000-2004, and an overview of trends since 1975. *Ann Oncol* 2010;21:1323-1360.

(4) Malvezzi M, Arfe A, Bertuccio P, Levi F, La VC, Negri E. European cancer mortality predictions for the year 2011. *Ann Oncol* 2011;22:947-956.

(5) Vogelstein B, Fearon ER, Hamilton SR et al. Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988;319:525-532.

(6) Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57-70.

(7) Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646-674.

(8) Algra AM, Rothwell PM. Effects of regular aspirin on long-term cancer incidence and metastasis: a systematic comparison of evidence from observational studies versus randomised trials. *Lancet Oncol* 2012;13:518-527.

(9) Chan AT, Ogino S, Fuchs CS. Aspirin use and survival after diagnosis of colorectal cancer. *JAMA* 2009;302:649-658.

(10) Langley RE, Burdett S, Tierney JF, Cafferty F, Parmar MK, Venning G. Aspirin and cancer: has aspirin been overlooked as an adjuvant therapy? *Br J Cancer* 2011;105:1107-1113.

(11) McCowan C, Munro AJ, Donnan PT, Steele RJ. Use of aspirin post-diagnosis in a cohort of patients with colorectal cancer and its association with all-cause and colorectal cancer specific mortality. *Eur J Cancer* 2013;49:1049-1057.

(12) Rothwell PM, Wilson M, Elwin CE et al. Long-term effect of aspirin on colorectal cancer incidence and mortality: 20-year follow-up of five randomised trials. *Lancet* 2010;376:1741-1750.

(13) Rothwell PM, Fowkes FG, Belch JF, Ogawa H, Warlow CP, Meade TW. Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. *Lancet* 2011;377:31-41.

(14) Rothwell PM, Wilson M, Price JF, Belch JF, Meade TW, Mehta Z. Effect of daily aspirin on risk of cancer metastasis: a study of incident cancers during randomised controlled trials. *Lancet* 2012; 379:1591-1601.

(15) Rothwell PM, Price JF, Fowkes FG et al. Short-term effects of daily aspirin on cancer incidence, mortality, and non-vascular death: analysis of the time course of risks and benefits in 51 randomised controlled trials. *Lancet* 2012.

(16) Davies M, Arumugam PJ, Shah VI et al. The clinical significance of lymph node micrometastasis in stage I and stage II colorectal cancer. *Clin Transl Oncol* 2008;10:175-179.

(17) Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* 2002;3:991-998.

(18) Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* 2004;21:137-148.

(19) Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. *Annu Rev Immunol* 2004;22: 329-360.

(20) Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol* 2006;6:836-848.

(21) Cavallo F, De GC, Nanni P, Forni G, Lollini PL. 2011: the immune hallmarks of cancer. *Cancer Immunol Immunother* 2011;60:319-326.

(22) Wischhusen J, Waschbisch A, Wiendl H. Immune-refractory cancers and their little helpers—an extended role for immunetolerogenic MHC molecules HLA-G and HLA-E? *Semin Cancer Biol* 2007; 17:459-468.

(23) Palmisano GL, Contardi E, Morabito A, Gargaglione V, Ferrara GB, Pistillo MP. HLA-E surface expression is independent of the availability of HLA class I signal sequence-derived peptides in human tumor cell lines. *Hum Immunol* 2005;66:1-12.

(24) Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nat Immunol* 2002;3:999-1005.

(25) Wischhusen J, Waschbisch A, Wiendl H. Immune-refractory cancers and their little helpers—an extended role for immunetolerogenic MHC molecules HLA-G and HLA-E? *Semin Cancer Biol* 2007; 17:459-468.

(26) Nosho K, Baba Y, Tanaka N et al. Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer, and prognosis: cohort study and literature review. *J Pathol* 2010;222:350-366.

(27) Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat Rev Immunol* 2006;6:715-727.

(28) Hokland M, Kuppen PJ. Natural killer cells: from "disturbing" background to central players of immune responses. *Mol Immunol* 2005;42:381-383.

(29) Menon AG, Morreau H, Tollenaar RA et al. Down-regulation of HLA-A expression correlates with a better prognosis in colorectal cancer patients. *Lab Invest* 2002;82:1725-1733.

(30) Liu F, Lang R, Zhao J et al. CD8(+) cytotoxic T cell and FOXP3(+) regulatory T cell infiltration in relation to breast cancer survival and molecular subtypes. *Breast Cancer Res Treat* 2011;130:645-655.

(31) Sandel MH, Speetjens FM, Menon AG et al. Natural killer cells infiltrating colorectal cancer and MHC class I expression. *Mol Immunol* 2005;42:541-546.

(32) Watson NF, Ramage JM, Madjd Z et al. Immunosurveillance is active in colorectal cancer as down-regulation but not complete loss of MHC class I expression correlates with a poor prognosis. *Int J Cancer* 2006;118:6-10.

(33) Popat S, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. *J Clin Oncol* 2005;23:609-618.

(34) Soreide K, Janssen EA, Soiland H, Korner H, Baak JP. Microsatellite instability in colorectal cancer. *Br J Surg* 2006;93:395-406.

(35) Dierssen JW, de Miranda NF, Ferrone S et al. HNPCC versus sporadic microsatellite-unstable colon cancers follow different routes toward loss of HLA class I expression. *BMC Cancer* 2007;7:33.

(36) Mouradov D, Domingo E, Gibbs P et al. Survival in stage II/III colorectal cancer is independently predicted by chromosomal and microsatellite instability, but not by specific driver mutations. *Am J Gastroenterol* 2013.

(37) de Bruin EC, van de Velde CJ, van de Pas S et al. Prognostic value of apoptosis in rectal cancer patients of the dutch total mesorectal excision trial: radiotherapy is redundant in intrinsically high-apoptotic tumors. *Clin Cancer Res* 2006;12:6432-6436.

(38) de Heer P, de Bruin EC, Klein-Kranenbarg E et al. Caspase-3 activity predicts local recurrence in rectal cancer. *Clin Cancer Res* 2007;13:5810-5815.

(39) Jonges LE, Nagelkerke JF, Ensink NG et al. Caspase-3 activity as a prognostic factor in colorectal carcinoma. *Lab Invest* 2001;81:681-688.

(40) Takano Y, Saegusa M, Ikenaga M, Mitomi H, Okayasu I. Apoptosis of colon cancer: comparison with Ki-67 proliferative activity and expression of p53. *J Cancer Res Clin Oncol* 1996;122:166-170.

(41) Kim GP, Colangelo LH, Wieand HS et al. Prognostic and predictive roles of high-degree microsatellite instability in colon cancer: a National Cancer Institute-National Surgical Adjuvant Breast and Bowel Project Collaborative Study. *J Clin Oncol* 2007;25:767-772.

(42) Okon K, Demczuk S, Klimkowska A et al. Correlation of microsatellite status, proliferation, apoptotic and selected immunohistochemical markers in colorectal carcinoma studied with tissue microarray. *Pol J Pathol* 2006;57:105-111.

(43) Dolcetti R, Viel A, Doglioni C et al. High prevalence of activated intraepithelial cytotoxic T lymphocytes and increased neoplastic cell apoptosis in colorectal carcinomas with microsatellite instability. *Am J Pathol* 1999;154:1805-1813.

(44) Gray RG, Quirke P, Handley K et al. Validation study of a quantitative multigene reverse transcriptase-polymerase chain reaction assay for assessment of recurrence risk in patients with stage II colon cancer. *J Clin Oncol* 2011;29:4611-4619.

(45) O'Connell MJ, Lavery I, Yothers G et al. Relationship between tumor gene expression and recurrence in four independent studies of patients with stage II/III colon cancer treated with surgery alone or surgery plus adjuvant fluorouracil plus leucovorin. *J Clin Oncol* 2010;28:3937-3944.

(46) Bastiaannet E, Sampieri K, Dekkers OM et al. Use of Aspirin postdiagnosis improves survival for colon cancer patients. *Br J Cancer* 2012.

(47) Ofosu FA. Appropriate Assessment of the Functional Consequences of Platelet Cyclooxygenase-1 Inhibition by Aspirin in vivo. *Thromb Res* 2013.

(48) Herschman HR. Prostaglandin synthase 2. *Biochim Biophys Acta* 1996;1299:125-140.

(49) Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol* 1971;231:232-235.

(50) Uddin S, Ahmed M, Hussain A et al. Cyclooxygenase-2 inhibition inhibits PI3K/AKT kinase activity in epithelial ovarian cancer. *Int J Cancer* 2010;126:382-394.

(51) Samuels Y, Wang Z, Bardelli A et al. High frequency of mutations of the PIK3CA gene in human cancers. *Science* 2004;304:554.

(52) Liao X, Lochhead P, Nishihara R et al. Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. *N Engl J Med* 2012;367:1596-1606.

(53) Labelle M, Begum S, Hynes RO. Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. *Cancer Cell* 2011;20:576-590.

(54) Reymond N, d'Agua BB, Ridley AJ. Crossing the endothelial barrier during metastasis. *Nat Rev Cancer* 2013;13:858-870.

(55) Placke T, Orgel M, Schaller M et al. Platelet-derived MHC class I confers a pseudonormal phenotype to cancer cells that subverts the antitumor reactivity of natural killer immune cells. *Cancer Res* 2012;72:440-448.

(56) Bruno A, Dovizio M, Tacconelli S, Patrignani P. Mechanisms of the antitumoural effects of aspirin in the gastrointestinal tract. *Best Pract Res Clin Gastroenterol* 2012;26:e1-e13.

(57) Chan AT, Ogino S, Fuchs CS. Aspirin use and survival after diagnosis of colorectal cancer. *JAMA* 2009;302:649-658.

(58) Liao X, Lochhead P, Nishihara R et al. Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. *N Engl J Med* 2012;367:1596-1606.

(59) Pepe MS, Etzioni R, Feng Z et al. Phases of biomarker development for early detection of cancer. *J Natl Cancer Inst* 2001;93:1054-1061.

List of publications

Prognostic implication of occult nodal tumor cells in stage I and II colon cancer. Sloothaak DAM, van der Linden R, Reimers MS, van de Velde CJH, Bemelman WA, Lips D, van der Linden JC, Doornewaard H, Tanis PJ, Bosscha K, van der Zaag ES, Buskens CJ, *submitted.*

Validation of the 12-gene colon cancer recurrence score as a predictor of recurrence risk in stage II and III rectal cancer patients. Reimers MS, Kuppen PJK, Lee M, Lopatin M, Tezcan H, Putter H, Clark-Langone K, Liefers GJ, Shak S, van de Velde CJH, *Journal of the National Cancer Institute* 2014 Sep 26;106(11).

Combined analysis of biomarkers of proliferation and apoptosis in colon cancer; an immunohistochemistry based study using tissue micro array. Reimers MS, Zeestraten ECM, van Alphen TC, Dekker JWT, Putter H, Saadatmand S, Liefers GJ, van de Velde CJH, Kuppen PJK. *International Journal of Colorectal Disease* 2014 Sep;29(9):1043-52.

Prognostic value of HLA class I, HLA-E, HLA-G and Tregs in rectal cancer: a retrospective cohort study. Reimers MS, Engels CC, Putter H, Morreau H, Liefers GJ, van de Velde CJH, Kuppen PJK. *BMC Cancer* 2014 Jul 5;14:486.

How does genome sequencing impact surgery. Reimers MS, Engels CC, Kuppen PJK, van de Velde CJH, Liefers GJ, *Nature Reviews Clinical Oncology* 2014 Oct;11(10):610-618.

The diagnostic value of One-Step Nucleic acid Amplification (OSNA) for sentinel lymph nodes in colon cancer patients. Vogelaar FJ, Reimers MS, van der Linden RLA, van der Linden JC, Smit VTHBM, Lips DJ, van de Velde CJH, Bosscha K., *Annals of Surgical Oncology*, 2014 Nov;21(12):3924-30.

Expression of HLA class I antigen, aspirin use, and survival after a diagnosis of colon cancer. Reimers MS, Bastiaannet E, Langley RE, van Eijk R, van Vlierberghe RL, Lemmens VE, van Herk-Sukel MP, van Wezel T, Fodde R, Kuppen PJK, Morreau H, van de Velde CJH, Liefers GJ, *JAMA Internal Medicine* 2014 May 1;174(5):732-9.

Clinical prognostic value of combined analysis of Aldh1, Survivin, and EpCAM expression in colorectal cancer. Goossens-Beumer IJ, Zeestraten ECM, Benard A, Christen T,

Reimers MS, Keijzer R, Sier CF, Liefers GJ, Morreau H, Putter H, Vahrmeijer AL, van de Velde CJH, Kuppen PJK, *British Journal of Cancer* 2014 Jun 10;110(12):2935-44.

Combined analysis of HLA class I, HLA-E and HLA-G predicts prognosis in colon cancer patients. Reimers MS, Zeestraten ECM, Saadatmand S, Dekker JWT, Liefers GJ, van den Elsen PJ, van de Velde CJH, Kuppen PJK. *British Journal of Cancer* 2014 Jan 21;110(2):459-68.

Biomarkers in precision therapy in colorectal cancer. Reimers MS, Zeestraten ECM, Kuppen PJK, Liefers GJ, van de Velde CJH, *Gastroenterology Report* 2013 Nov;1(3):166-83.

The prognostic value of the apoptosis pathway in colorectal cancer: a review of the literature on biomarkers identified by immunohistochemistry. Zeestraten ECM, Benard A, Reimers MS, Schouten PC, Liefers GJ, van de Velde CJH, Kuppen PJK, *Biomarkers in Cancer* 2013 Juli 4;5:13-29.

Aspirin use after diagnosis improves survival in older adults with colon cancer: a retrospective cohort study. Reimers MS, Bastiaannet E, van Herk-Sukel MP, Lemmens VE, van den Broek CB, van de Velde CJ, de Craen AJ, Liefers GJ, *Journal of the American Geriatrics Society*, 2012 Dec;60(12):2232-6.

Een enterocutane fistel: het gevolg van een gecompliceerd beloop van een penetrerend abdominal trauma. Reimers MS, Kroon HM, Neijenhuis PS, Schipper IB, *Nederlands Tijdschrift voor Heelkunde*, December 2011.

List of co-authors

Tert C. van Alphen, Leiden University Medical Center, The Netherlands. Department of Surgery.

Esther Bastiaannet, Leiden University Medical Center, The Netherlands. Department of Surgery and Department of Geriatrics and Gerontology.

Colette B. M. van den Broek, Leiden University Medical Center, The Netherlands. Department of Surgery.

Kim Clark-Langone, Genomic Health Inc., Redwood City, California, USA.

Anton J. M. de Craen, Leiden University Medical Center, The Netherlands. Department of Geriatrics and Gerontology.

Jan-Willem T. Dekker, Leiden University Medical Center, The Netherlands. Department of Surgery.

Ronald van Eijk, Leiden University Medical Center, The Netherlands. Department of Pathology.

Peter J. van den Elsen, Leiden University Medical Center, The Netherlands. Department of Immunohematology and Blood Transfusion. VU University Medical Center, The Netherlands. Department of Pathology.

Charla C. Engels, Leiden University Medical Center, The Netherlands. Department of Surgery.

Riccardo Fodde, Erasmus Medical Center, The Netherlands. Department of Experimental Pathology.

Myrthe P. P. van Herk-Sukel, PHARMO Institute for Drug Outcomes Research, The Netherlands.

Peter J. K. Kuppen, Leiden University Medical Center, The Netherlands. Department of Surgery.

Ruth E. Langley, Medical Research Council Clinical Trials Unit at University College London, UK.

Mark Lee, Genomic Health Inc., Redwood City, California, USA.

Valery E. P. Lemmens, Comprehensive Cancer Center South, The Netherlands.

Gerrit Jan Liefers, Leiden University Medical Center, The Netherlands. Department of Surgery.

Margarita Lopatin, Genomic Health Inc., Redwood City, California, USA.

Hans Morreau, Leiden University Medical Center, The Netherlands. Department of Pathology.

Hein Putter, Leiden University Medical Center, The Netherlands. Department of Medical Statistics.

Sepideh Saadatmand, Leiden University Medical Center, The Netherlands. Department of Surgery.

Steve Shak, Genomic Health Inc., Redwood City, California, USA.

Haluk Tezcan, Genomic Health Inc., Redwood City, California, USA.

Cornelis J.H. van de Velde, Leiden University Medical Center, The Netherlands. Department of Surgery.

Ronald L. P. van Vlierberghe, Leiden University Medical Center, The Netherlands. Department of Surgery.

Tom van Wezel, Leiden University Medical Center, The Netherlands. Department of Pathology.

Eliane C.M. Zeestraten, Leiden University Medical Center, The Netherlands. Department of Surgery.

Curriculum Vitae

Marlies Suzanne Reimers was born on April 2nd 1984 in Dordrecht, The Netherlands. After graduating from the Johan de Witt Gymnasium in Dordrecht in 2002, she studied Bio-Pharmaceutical Sciences at the Leiden University for one year, before she started Medical School at the Leiden University Medical Center in 2003. After completing her preclinical training in medicine, she started with her clinical rotations in December 2007. During her rotations, she has spent two months at the Manguzi Hospital in Manguzi, South Africa. After obtaining her medical degree in November 2009, she started working at the department of surgery of the Rijnland Hospital, Leiderdorp, under supervision of Drs. S.A. da Costa, surgeon. In April 2011 she started her PhD research project that resulted in the current thesis under supervision of Prof. dr. C.J.H. van de Velde, Dr. G.J. Liefers and Dr. P.J.K. Kuppen. In July 2014, Marlies has started her residency training in radiology at the Albert Schweitzer Hospital in Dordrecht and the Erasmus Medical Center in Rotterdam under supervision of Drs. T.R. Hendriksz.

Dankwoord

Nu mijn proefschrift klaar is, wil ik graag iedereen bedanken die nauw betrokken is geweest bij het tot stand komen van dit proefschrift.

Prof. dr. C.J.H. van de Velde; het was een eer om deel uit te hebben gemaakt van uw onderzoeksgroep en om uw expertise op oncologisch gebied te ervaren.

Dr. G.J. Liefers, beste GJ; voor mij ben jij, met je onvermoeibare enthousiasme voor onderzoek en je scherpe klinische interpretatie van onze geproduceerde data op het lab, een groot voorbeeld. Ik wens je nog heel veel succes met ons 'kindje' (de Aspirin Trial). Hopelijk zorgt jouw inzicht ervoor dat over 10 jaar aspirine wordt gebruikt bij de behandeling van darmkanker patiënten.

Dr. P.J.K. Kuppen, beste Peter; bedankt dat je altijd klaar stond om te discussiëren over onze resultaten. Met name op het gebied van tumorimmunologie was je een inspiratiebron.

Mijn paranimfen Charla en Noor; Charla, mijn partner in crime; wat ben ik blij dat ik samen met jou dit traject heb kunnen doorlopen en daarnaast lekker gek heb kunnen doen op de P-01. Sinds het eerste moment dat je begon waren we onafscheidelijk. Zonder jou was het een stuk lastiger geweest. Ik zal onze samenwerking missen, maar ik weet zeker dat onze vriendschap zal blijven bestaan. Noor, we kennen elkaar al ons hele leven en hoe speciaal is het dat jij mijn paranimf wilt zijn. Dank je wel dat ik altijd bij je terecht kon om mijn hoofd leeg te maken en voor de gezellige afleiding die jij mij altijd hebt gegeven.

Gabi, captain van de kamer; wat was het altijd gezellig en wat heb ik veel aan je gehad! Dank dat ik altijd bij je terecht kon voor al mijn vragen.

De gehele onderzoeksgroep van de chirurgische oncologie, met name de meisjes van de D6; Wat was het fijn om deel uit te maken van zo'n leuke, gezellige, inspirerende en hard werkende groep. De samenwerkingen hebben geresulteerd en resulteren in prachtige publicaties. Ik ga jullie missen.

Dit proefschrift is alleen tot stand gekomen op basis van samenwerking en dankzij hulp van velen. Mijn dank is dan ook groot aan alle co-auteurs, collega's van de pathologie, collega's van lab heelkunde (in het bijzonder Ronald, Rob, Connie en Geeske), het datacenter en studenten. Bedankt voor de fijne samenwerking.

Vrienden en familie; dank voor al jullie steun en betrokkenheid bij het tot stand komen van dit proefschrift. In het bijzonder mijn ouders, zus en zwager; dank voor jullie geloof in mij.

Last but not least, lieve Hidde; ik ben je ontzettend dankbaar voor je geduld, liefde en steun de afgelopen jaren.