General Discussion and Future Perspectives

General Discussion
The treatment of GI cancers and in particular that of gastric, colon and rectal cancer is multidisciplinary and requires close collaboration between surgeons, gastroenterologists, radiologists, pathologists, radiation therapists, medical oncologists, oncologic nurses and psychologists. Surgical tumor removal is the mainstay of GI cancer treatment. The surgeon therefore plays a central role in coordinating the various treatment options after the initial diagnosis. Biomarkers may improve guiding of such treatment decisions. Postoperatively, after complete pathologic disease staging, the current markers in GI cancer (i.e. pTNM stage, CT/PET-imaging) are adequate and remain our best tools in the shed. Patient disease recurrence and survival data, however, indicate that there is much room for improvement in GI cancer staging, especially for early disease patients\(^1\)\(^-\)\(^3\). Also, there is a need for biomarkers that can guide preoperative treatment. One must keep in mind that TNM-staging is an observer-dependent, anatomical and therefore surrogate marker of cancer that does not include primary cancer cell properties. Hence, there is a need for a new category of GI cancer biomarkers. Primary tumor cell molecular features, assessed in preoperative biopsy material, are a very attractive tool which is currently under investigation. Molecular oncologic scientists are increasingly appreciating the role of epigenetic factors in GI carcinogenesis. Epigenetic primary tumor biomarkers, such as DNA methylation therefore merit evaluation to assess their possible utility to aid GI cancer treatment\(^4\)\(^-\)\(^6\). This thesis studies aimed at two goals: to further develop analytical techniques for DNA methylation assessment, and secondly, to validate DNA methylation markers in retrospective studies using primary GI tumor tissue. The epigenetic biomarkers’ value was tested for predicting disease outcome parameters that are of specific use in GI cancer treatment.

Section 1: Technical Advances
In chapter one a method is presented that helps to control methylation-specific PCR (MSP) analysis of DNA samples. As a basic principle in scientific experiments, each performed assay needs to be positively and negatively controlled to allow proper, undisputed interpretation of a tested sample’s result. To obtain positive controls in MSP assays means that in each experiment a sample should be amplified which contains DNA verified to have the target sequence fully methylated. Such DNA can be obtained by incubating whole genome human DNA with SssI (Spiroplasma sp. Strain MO1) methyltransferase in the presence of S-adenosylmethionine (SAM) as a methyl-group donor\(^7\). To obtain negative controls for MSP experiments verified to have the target sequence unmethylated is very time-consuming and sometimes impossible. Mostly, DNA from peripheral blood lymphocytes is used in which usually genes of interest in cancer research show to be unmethylated. However, some genes are methylated in the healthy human genome. The first section describes a protocol to synthesize large quantities of completely unmethylated genomic DNA that can be used as a negative control in any methylation experiment, a so-called universal unmethylated control. The method is
based on that phi-29 DNA polymerase, an enzyme used for whole genome amplification does not transfer methylation patterns to its synthesized copies. After several rounds of amplification the amount of possibly methylated start material DNA becomes undetectable for PCR and completely unmethylated DNA is obtained. This method is cheap, useful and can be performed in any standard equipped laboratory.

In chapter two, a protocol for DNA preparation for methylation analysis is presented that is well suitable for tissue resources such as formalin fixed, paraffin-embedded (FFPE) tissue blocks derived from pathology department’s archives. Although suboptimal for DNA evaluation, this specimen resource is highly valuable for initial retrospective assessment of biomarkers as these tissue blocks are abundantly available and long term follow up data of the patient’s disease outcomes can be retrieved. Also, hematoxylin-eosin stained sections cut from FFPE tissue blocks provide excellent assessment of tumor tissue histopathology and is the golden standard. Some basic limitations have to be taken into account when working with DNA derived from FFPE tissue compared to more optimal DNA resources, i.e. from fresh frozen tissue. The formalin incubation the tumor tissue before fixation in paraffin causes DNA cleavage which results into a reduced average DNA strand length that hampers PCR amplification depending on the chosen amplicon length. Protocols to purify DNA for PCR preparation target to lose contaminating, PCR inhibiting proteins and salts. However, during purification there is much collateral loss of sample DNA. The further needed bisulfite modification (SBM) treatment again reduces and degrades the amount of sample DNA as it is incubated at 60 degrees for three hours at a pH of 5. After SBM, several steps are needed to purify the DNA from the sodium bisulfite salts that will interfere with reaction buffers and hamper Taq-polymerase activity. chapter two describes the on-slide SBM protocol that was designed to reduce the number of steps needed to work up DNA for methylation status assessment. The protocol was adapted from a study by Nuovo et al. After deparaffinization, the tissue section on a glass slide is directly incubated in bisulfite solution with the DNA remaining in-situ, in the cell’s nucleus (see figure 1). Our study at first compared this method with the classic used methods for bisulfite modification and shows that on-slide SBM converts DNA as efficiently as the standard protocols while harvesting significantly higher amounts of DNA. The on-slide modification dramatically reduces DNA loss as it eliminates
purification steps as the DNA remains in-situ. Subsequently, on-slide SBM enables a high MSP success rate of tissue areas as small as 1-2 square millimeters of 7 µm tissue sections. Other studies ensure a sufficient yield of modified sample DNA for PCR by increasing the amount of start sample. Sometimes this is achieved by putting in several whole paraffin tissue sections or lysis of complete fresh tumor tissue cubes. These methods compromise on accuracy as input DNA is not controlled for actual tumor cell content.

In the diagnostic work-up of the pathologist, usually the invasive margins of a tumor are assessed for T-stage and often in these transitional areas cells of the precursor lesion can be identified and also normal mucosa. An application of on-slide SBM is that DNA methylation status can be compared between malignant and adjacent pre-malignant and normal tissue within the same tissue section with paired analysis statistics. This is important as in other studies comparisons are often made between non-paired groups of cancer and adenoma specimens. The potential to develop into an invasive lesion is unsure when using adenoma tissue from a polyp harvested by colonoscopy. On-slide SBM therefore ensures that the adenoma represents a precursor lesion that has a proven progression into invasive carcinoma. To exemplify application, it was demonstrated in colorectal cancer that promoter region hypermethylation of the important tumor-related genes RASSF1a and p16/INK4a occurs in the cancer stage and not in the adenoma stage. When applied in breast cancer i.e. ductal carcinoma in-situ (DCIS) areas can be compared with invasive ductal carcinoma, in cervical cancer i.e. cervical intraepithelial neoplasia (CIN) can be compared with squamous cell carcinoma. On-slide SBM can increase our knowledge of DNA methylation differences between cancers and their precursor lesions with optimal tissue histopathologic control. It may help to elucidate the changes that are specific for the transition between benign adenoma to invasive carcinoma which is the key step in malignant tumor formation.

For DNA methylation analysis our group uses capillary array electrophoresis MSP (CAE-MSP) as a standard technique for methylation analysis. CAE-MSP has several advantages over the classic MSP assay that uses agarose gel electrophoresis for PCR product detection. Gel electrophoresis is operator dependent and often requires nested PCR to obtain detectable bands which may induce non-specific amplification results. In CAE-MSP, a single round of PCR is performed with color-labeled, automatically detectable oligonucleotides with different colors for the methylation specific and unmethylation specific reaction. This enables sensitive amplicon detection in a high through-put capillary automated detection system using 96-well plates followed by automated software fragment analysis. The procedure also allows semi-quantitative interpretation of the data and confirms with each sample the predicted amplicon size length with high precision. As mentioned, CAE-MSP has excellent qualities, however, it is not a fully quantitative assay. In chapter three, absolute quantitative assessment of methylated alleles (AQAMA) is introduced for accurate assessment of a sample’s DNA methylation level instead of methylation status. The AQAMA PCR principle was derived from QAMA described by Zegsnik et al. and is based on real-time PCR techniques. In AQAMA, two fluorescent minor-groove binding (MGB) probes, one methylation-specific and one unmethylation-specific are used to detect the amount of methylated and unmethylated alleles present in sample DNA. Important features of AQAMA that enhance reliable quantification are: 1. The use of minor groove binding (MGB) probes ensures the required high specificity to discrimi-
nate between CT (unmethylated) and CG (methylated) SBM modified sequences\textsuperscript{17-19}. 2. Quantification is performed in a single PCR reaction while other methods use separate methylation specific and unmethylation specific reactions with different reaction kinetics. 3. The use of standard curves of dilution series of cDNA synthesized in E.Coli vectors enables absolute copy number assessment of methylated and unmethylated alleles with highly specific positive and negative control of the quantification. 4. The assay can be performed in a 384 wells system that further reduces inter-assay variability when dealing with large sample sizes as our studies intend. In \textbf{chapter three} the quantitative accuracy and linearity of AQAMA is demonstrated and its application by combining AQAMA with on-slide SBM. Tissue areas with different histopathology could be quantitatively compared for differences in methylation level instead of dichotomous comparison of methylated versus unmethylated. As an example significant differences in level of MINT locus methylation (a known specific marker for colorectal cancer) were detected between adenoma and cancer colorectal tissue. AQAMA combined with on-slide SBM can provide further quantitative detail of epigenetic events during colorectal or other solid tumor formation.

The onslide-SBM technique majorly improves and simplifies DNA preparation for DNA methylation analysis. As cells are harvested by needle scratch under a light microscope, it is inevitable that the finally analyzed sample contains some normal cells such as tumor infiltrating lymphocytes or stromal cells. To further reduce non-tumor cells sample content and to strive for analyzed DNA to be derived from 100% tumor cells, on-cap SBM was developed as described in \textbf{chapter four}. The method integrates in-situ SBM with the laser capture microdissection platform\textsuperscript{20,22}. The cells stick to a cap that can be lifted from the section and afterwards the cells can be treated, \textit{in-situ}, with sodium bisulfite before cell digestion and continued DNA isolation. We combined on-cap SBM with the AQAMA assay to measure global hypomethylation of colorectal cancers by targeting long interspersed nucleotide element one (LINE-1), a DNA repeat that represents about 15% of the human genome. On-cap SBM followed by AQAMA of LINE-1 allowed as a first to demonstrate stepwise reduction of global hypomethylation in colorectal cancer with advanced disease stage. We also showed necessity of employing target cell selection techniques, such as laser capture microdissection, when analyzing LINE-1 in colorectal cancer.

\textbf{Section 2: Clinical application}

These thesis projects were initiated because of an interest to test clinical utility of epigenetic biomarkers using patient tissue of clinical databases from trials coordinated by the Leiden University Medical Center Surgical Department. Two trials were selected: the first concerned gastric cancer, the so-called D1D2 trial, and the other concerned rectal cancer, the so-called total mesorectal excision or TME-trial. The studies were initiated by the Dutch Gastric Cancer Group and the Dutch Colorectal Cancer Group, respectively. These collaborative scientists’ networks have a unique infrastructure covering almost every oncologic surgical clinic in the Netherlands enabling a nation-wide cohort inclusion of GI cancer patients. As almost every citizen in the Netherlands has a family physician, patients can easily be tracked after clinical discharge which enhances complete long-term follow-up data. The clinical results of primary endpoints of these trials have been published\textsuperscript{23,24} and primary tumor tissue blocks were col-
lected from the different study sites pathology departments. As a consequence, the patients of the study groups in this thesis were very well documented with prospectively collected follow-up data.

The D1D2 trial compared total or partial gastric resection with D1 lymph node dissection to surgery with more extensive D2 lymph node dissection that is widely used in Asian countries and is mostly promoted by the expert gastric cancer surgeons from Japan. Almost 1000 patients were randomized in this study providing a unique patient group with a median of 14 years of follow-up at the time of our biomarker data analysis. As discussed in this thesis introduction, the multidisciplinary treatment of gastric cancer has long been frustrated by inability to show effect of (neo)adjuvant chemotherapeutics\textsuperscript{25}. A landmark study by McDonald et al. in 2001 was the first to show benefit of postoperative chemoradiotherapy and again gave an impulse to put scientific effort in improving treatment of gastric cancer\textsuperscript{26}. In 2006, Cunningham et al. showed benefit of perioperative chemotherapy over surgery alone\textsuperscript{27}. These important studies have created a need for biomarkers in gastric cancer to better allocate patients to the introduced aggressive treatment regimens. *Cyclo-oxygenase-2 (COX-2)* is a well studied gene in many cancers and predominantly in GI cancer. The COX-2 enzyme is involved in production of prostaglandine PGE\textsubscript{2} and to a lesser extent thromboxane, both implicated in angiogenesis, cell proliferation, cell survival, migration, invasion, and modulation of host immune cells\textsuperscript{28-30}. Tumors overexpressing COX-2 have been shown to behave more aggressively during clinical follow-up in gastric cancer as well as other cancers\textsuperscript{31,32}. The epigenetic regulation of COX-2 has been described in gastric cancer\textsuperscript{33-35}. The methylation status of the COX-2 promoter region has not been tested to have utility as a biomarker in gastric cancer. In chapter 5 it was demonstrated that a hypermethylated status of the COX-2 promoter region indeed leads to underexpression of COX-2 protein and subsequently to improved disease survival. A strong aspect of this study was that the predictive value was shown in two independent clinical trial populations which reduces the risk of overfitting of the data. The predictive value was independent of the established clinical parameters and therefore may have value to further aid treatment decisions in gastric cancer. From this study's conclusion it can be derived that epigenetic silencing does not necessarily result into more aggressive disease outcome of gastric cancer. DNA methylation research generated much interest because it was novel way of shut-down of tumor suppressor genes as a cause of carcinogenesis. This has lead to a paradigm that DNA methylation is an unfavorable tumor event. Since demethylating drugs are available there is an argument that they may be used to remove those unwanted methyl-groups and make the tumor-suppressor genes function again. In chapter 5 we demonstrate DNA methylation to have a favorable role in predicting disease outcome and removal of this methylation by demethylating drugs may therefore worsen disease outcome. The data further suggest that specific gene promoter region hypermethylation may replace gene protein expression as a biomarker. DNA methylation assays have some specific advantages (i.e. non-receiver-operated, automated) and may be used instead of assessment of protein expression by immunohistochemistry when that gene is epigenetically regulated.

The Dutch TME trial on rectal cancer was the second clinical study of interest to test DNA methylation biomarker utility. The study showed improvement of the surgical treatment of rectal cancer by reducing local recurrence rates by adding short course radiotherapy befo-
re surgery\textsuperscript{23}. An important quality of this trial was the standardization of the surgical procedure using the total mesorectal excision technique described by Heald et al\textsuperscript{36}. Participating surgeons underwent a technical training program and were supervised during the first five operations. Furthermore, standardization of the pathology assessment of the rectal specimens controlled for completeness of the surgical treatment. The trial included 1861 patients in less than 4 years from over 108 surgical clinics. To date, epigenetic biomarkers have not been tested in rectal cancer specifically. For the study described in chapters 6 and 7, we established research objectives that are of specific use for rectal cancer treatment such as prediction of local recurrence and distant recurrence in early disease. In the experiments testing AQAMA and on-slide SBM we obtained encouraging data that methylation levels may have potential to subclassify rectal cancers. The required sample size for showing prognostic value was established by sample size calculations. The four MINT loci used in the AQAMA validation studies (MINT1, 2, 12 and 31) were used as markers and expanded by three more (MINT3, 17 and 25). To test if change in MINT methylation levels was related to tumor progression we first analyzed normal, adenoma and cancer tissues (chapter 6). We used the assay combining on-slide-SBM and AQAMA to study this. The data showed that MINT locus methylation establishes during adenomatous transformation of normal rectal epithelium and is maintained during malignant transformation. Biomarkers in other studies often positively correlate to increasing TNM-stage meaning that only a small number of cases with early disease are positive. Such markers are therefore less likely to be of clinical use in early stage patients while this group would benefit most from better subclassification. The clearly detectable and non-normally distributed MINT methylation levels in adenomas and early cancer were therefore promising to subclassify early stage disease. Then MINT locus methylation values were measured in 314 non-irradiated patients’ tissues. Unsupervised random forest clustering of the data was used to analyze whether specific methylation patterns could be discerned from the data. This unconventional statistical method was opted for because of its capacity of internal validation, eliminating the need of a test-validation study set-up. Random forest (RF) clustering does not make assumptions on data distribution which makes it suitable for analyzing the non-normally distributed methylation data. Two methylation markers (MINT3 and MINT17) were identified by RF clustering that could separate the patients into four groups. Importantly, the separation was clear, with no overlap. Most biomarkers concern gene expression data with a cut-off being the median expression value. Because gene expression data are usually normally distributed, separation by this cut-off is not clear. Interestingly, an inverse relation between MINT3 and 17 methylation was present and this specific combination was required to have predictive value for distant recurrence probability in early, node-negative disease. This predictive value was not present in node-positive disease. Disease spread early by the hematogenic route may explain this finding. There is evidence of systemic spread of colorectal cancer cells in the circulation and bone marrow even in the earliest stages\textsuperscript{37-39}. This indicates that very early on, tumor cells can have the capacity to enter to bloodstream and therefore may metastasize without signs of lymphatic spread. Our assay may have identified rectal tumors with such early potential for hematogenic spread. Further studies are needed to support this.

The MINT3 locus CpG island is localized on chromosome 1p34-35 just downstream of RBBP4 (retinoblastoma-binding protein 4\textsuperscript{45,46}), SYNC1 (encoding syncoilin protein involved in cell cytoskeleton and extracellular matrix proteins), YARS (tyrosyl-tRNA synthetase, involved
in angiogenesis\textsuperscript{47,48} and s100p-binding protein (s100p is known to be overexpressed in many solid tumors\textsuperscript{49,50}). MINT17 is localized on the long arm of chromosome 12 upstream of the Harakiri (HRK) gene which is a member of the BCL2 gene family that encodes apoptosis regulatory proteins and its expression is a known target of regulation by promoter region methylation\textsuperscript{51}. A functional relation between MINT3 and 17 and the mentioned genes or their expressed proteins has not been established but is an interesting subject of future studies.

5-Fluoro-uracil (5FU) -based adjuvant treatment for rectal cancer is given standard in the United States and Asia for transmural (T3) and/or node-positive rectal cancer\textsuperscript{40,41} and the SCRIPT trial (www.dccg.nl) currently investigates adjuvant treatment with capecitabine (an oral form of 5FU) for this patient group in the Netherlands. The clinical utility of predicting distant recurrence in early stage rectal cancer patients becomes now important since clinicians may be hesitant to consider node-negative patients for adjuvant therapy as it is known that only 15-20 percent of these patients will show distant recurrence. Together, our results were shown in a large trial-derived patient group that was controlled for surgical and pathological quality and that was established by sample size calculations. We used preselected markers with shown potential for subclassification and obtained clearly distinct subgroups in an unbiased manner by using unsupervised random forest clustering. The predictive value in early disease patients may have utility in targeting systemic treatment after surgery due to identification of high risk on distant disease spread despite the early detection.

Another important clinical issue in the rectal cancer specifically is the risk of local recurrence due to the close anatomical relation of the rectum to the pelvis. Rectal cancer patients are therefore additionally treated with radiotherapy pre- and/or intraoperatively to improve local control. Radiation therapy in addition to surgery comes with specific long term morbidity such as more faecal incontinence, sexual dysfunction and a general reduced quality of life\textsuperscript{42-44}. Local recurrence rates after adequate TME surgery alone are around 10% meaning that 90% will be overtreated when all rectal cancer patients undergo short-course preoperative radiotherapy. Targeting radiation treatment to those patients who are likely to locally recur could therefore be of benefit. In chapter 7 the predictive value of MINT3 and MINT17 epigenetic biomarkers for local recurrence was evaluated. The specific combination of hypermethylation of MINT3 and decreased methylation of MINT17 was tested to be predictive for this purpose. This patient group which was at high risk for distant recurrence had significantly lower risk for local recurrence. In chapters 6 and 7 the identification of an epigenetically distinct group of rectal cancer patients who early on in their disease formation have increased potential metastasize systemically without local spread is reported. These patients may benefit more from systemic chemotherapy and less from preoperative radiation. It is important to notice that since MINT3 and MINT17 primary tumor tissue methylation levels as biomarkers can be assessed preoperatively on tumor biopsy material so it can be considered in the preoperative multidisciplinary decision making process.

In chapter 8 the relation between quantitative MINT locus methylation and microsatellite instability (MSI) is analyzed. MSI+ colorectal cancers are a firmly established genetically distinct subgroup of sporadic colorectal cancers with mismatch repair system defectiveness and clear guidelines exist for assays to identify MSI+ cases. Sporadic MSI occurs in approximately 15% of colorectal cancers and 50% of right-sided colon cancers. Studies
have repeatedly shown a relation between DNA methylation and MSI. It is now accepted that sporadic MSI occurs through epigenetic silencing by DNA methylation of the important mismatch repair protein \textit{hMLH1}. This is observed in tumors with genome-wide, global increased methylation at multiple CpG islands\textsuperscript{52}. Still many issues remain unsolved. For instance at what stage of tumor formation (normal, adenoma or cancer) the relation between globally increased methylation, methylation of \textit{hMLH1} and MSI is initiated. Our assay of on-slide-SBM combined with AQAMA is suitable to answer this specific question as paired analysis of quantitative methylation and genetic MSI analysis can be performed in different stages of CRC development. The results showed increased methylation of MINT loci and \textit{hMLH1} in adenomatous precursors of MSI+ CRCs which showed to be MSI+ in only 50% of the cases. The data indicate global hypermethylation to precede MSI at the adenoma stage of tumor formation. Inconsistent results are published on whether MSI+ CRCs have significant better disease survival. \textit{hMLH1} methylation is causative of sporadic MSI and this methylation is part of a global, genome-wide increased methylation of which, according to our results, MINT loci are representative. Our results show that level of MINT locus methylation has predictive value for disease outcome independent of MSI status. This result is confirmed by other groups\textsuperscript{53-55}. The assumed improved outcome of MSI+ tumors may therefore be explained by increased MINT locus methylation levels. How high MINT locus methylation levels improve disease survival or how low MINT locus methylation enhance distant metastasis is a subject for future studies.

There are many studies that have suggested colorectal cancers with heavy methylation at CpG islands to form a clearly distinguishable subgroup of colorectal cancers, even a phenotype, the CpG island methylator phenotype (CIMP). Clinically, CIMP+ tumor features overlap with those of MSI+ tumors which are: mucinous aspect, proximal location in the large bowel and poor differentiation\textsuperscript{55}. Genetically, there is a close association with BRAF V600E mutations and KRAS wild type\textsuperscript{52,56}. How to define CIMP+ cases is not agreed upon as is in MSI. Different groups use different markers and different assay techniques (often non-quantitative\textsuperscript{53}). The frequently used quantitative MethyLight technique\textsuperscript{56} sometimes does not measure unmethylated allele presence and normalizes the methylated allele number to a total allele count which may lead to underestimation of the degree of methylation since DNA methylation is known to be heterogeneous. Also, the quantitative data are dichotomized by a cut-off into methylated or unmethylated and the actual methylation level value is never used in clinical correlation studies. The cases showing hypermethylation in chapter 8 likely overlap with CIMP+ patients. Our study ’s objective, however, was not to test whether CIMP exists, which has been established. We intended to study the quantitative relation between methylation of MINT loci and MSI+ in normal, premalignant and malignant colorectal tissue.

\textbf{Concluding Remarks and Future Perspectives}

\textbf{Techniques}

In section one of this thesis, on-slide SBM, AQAMA and on-cap SBM are presented as techniques for detailed quantitative epigenetic assessment of cancer specimens. These assays are relatively simple to perform. On-slide SBM requires no more equipment than needed
for standard immuhistochemical staining protocols. AQAMA requires a single PCR reaction on an automated real-time PCR platform. We showed its quantitative quality, its application and its potential clinical value. When interpreting results from other studies that evaluate epigenetic biomarkers consideration should be given to the techniques used. Quantitative PCR analysis of primary tumor tissue has no role in GI cancer diagnostics yet. The results of this thesis’ studies show promising prospects for clinical use of AQAMA in GI cancer diagnostics. The quantitative detail of DNA methylation is yet to be further tested in other GI malignancies and solid tumors for clinical utility.

The on-slide or on-cap technique combined with AQAMA has potential to elucidate the relation of epigenetic and genetic events in the carcinogenic process of any solid tumor. This thesis’ studies utilized the techniques in colorectal cancer, although the technique can compare histologically different tissue areas within a single tissue section of any type of solid tumor. The approach enabled to clarify that the relation between microsatellite instability and DNA methylation is established in the earliest stages in the oncogenic pathway of colorectal cancer. The epigenetic aspects of the histopathologically important steps in tumor formation of premalignant transformation of normal cells and invasive transformation of premalignant cells can be better studied with on-slide SBM. Applicable to any type of cancer, the opportunities of new study subjects are numerous and likely to yield interesting results.

**Biomarkers**

Epigenetics, like DNA methylation form a new category of molecular targets for biomarker opportunities. This thesis’ studies aimed at testing DNA methylation to have utility in clinically relevant subclassification of GI cancers. The data demonstrated that specific epigenetic biomarkers in primary tumor tissue of gastric, colon and rectal cancer (the most common forms of GI cancer) can identify clearly separable subgroups of GI cancer patients and to have potential aid in making preoperative treatment decisions guiding the multidisciplinary treatment. To date, the TNM staging system is the best available tool to base treatment decisions upon and will continue to do so in the future. We show that quantitative epigenetic biomarkers can give valuable additional information in assessment of primary tumor properties for stratification of disease aggressiveness.

*COX-2* methylation status was identified in this thesis’ studies in gastric cancer to have prognostic value independent from TNM-stage. Only recently, effective (neo)adjuvant treatment regimens are being administered in gastric cancer. Further clinical studies have to point out what subgroups of gastric cancer patients will benefit most by these aggressive treatment regimens and biomarkers, like *COX-2* methylation analysis, will likely be of additional value. Confirmative studies are needed to test *COX-2* prognostic value in node-negative disease specifically. These analyses should be performed with a fully quantitative assay as our studies were performed with semi-quantitative techniques.

In rectal cancer we show that quantitative assessment of two MINT loci in primary tumor tissue can be of aid in specific clinical problems. Preoperative treatment planning is becoming more and more important since neoadjuvant strategies have been shown to be effective. MINT locus methylation assessed by AQAMA can help select patients for preoperative radiation therapy and similar as in colon cancer can help identify early stage, high-risk disease that may benefit from systemic treatment reducing distant metastasis. The fin-
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Figure 2: Suggested trial randomization schedule using MINT3 and MINT17 as biomarkers in non-locally advanced rectal cancers.

...dings need to be validated in an independent prospectively collected patient group. If confirmed, the results may be used to design clinical trials that stratify non-locally advanced patients into high-risk local / low-risk distant recurrence or low-risk local / high-risk distant recurrence and tailor treatment accordingly (see figure 1). In the future rectal cancer should be evaluated in biomarker studies as a separate group of large bowel cancers because it has specific treatment and specific clinical aspects to be taken into consideration.

In colon cancer, there is much discussion how to improve the selection of high-risk node negative patients for adjuvant chemotherapy. In node negative disease, primary tumor properties are becoming of clinical importance. The pathology report now additionally describes whether a tumor has signs of perineural/vascular/lymphatic invasion, however, these assessments are not routinely and systematically performed. Our studies show that MINT locus methylation is especially suitable in node-negative disease when it is detected in early disease, even at the adenomatous stage. These epigenetic biomarkers may therefore be of specific help in identifying high-risk, node-negative colon patients. Validation studies in independent, prospectively collected patient groups are needed to further investigate this.

Finally, this thesis is an example that shows the worth of close collaboration between surgical oncologists and basic scientists and demonstrates that translational medicine is of great value in cancer research to test clinical application of novel molecular targets and techniques and subsequently bring the so-called “bench” and “bedside” closer to each other.
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