General introduction
Gastrointestinal cancers
The lineage of the lumen of the gastrointestinal (GI) tract from mouth to anus consists of mucosal cells that separate the inner world of the body from the outer environment. The functions of these cells are numerous as well as diverse: active uptake of nutritional elements, passive passage of fluids and electrolytes, secretion of digestive enzymes, defense from pathogenic bacteria, facilitating stool transportation etcetera. To enhance its performance and exchange capacity the mucosal surface is increased by extensive folding on the macroscopic level: i.e. plicae gastricae of the stomach, Kerckring’s folds in the small bowel, haustrae of the large bowel and on the microscopic level: i.e. gastric foveolae and colonic crypts and villi. If spread out, the total surface of the digestive tract would be the size of approximately two tennis courts. The regenerating capacity of the GI tract lineage is tremendous; i.e. the mucosal layer of the large intestine completely renews itself every 5 days. The intensive interaction with outer environment and the high rate of cell division may contribute to the fact that cancers of the gastrointestinal tract are among the most widely seen in humans. In 2008 in the United States, 271,290 new cases of GI cancer were diagnosed accounting for 19% of all cancers and GI cancers caused 24% of all cancers deaths (see table 1)\(^1\).

Causative genetic mutations have been identified in some hereditary forms of GI cancers such as Lynch syndrome\(^2-5\) (former hereditary non-polyposis colorectal carcinoma, HNPCC) and familial adenopolyposis (FAP)\(^6\). The exact cause of sporadic cancers of the GI tract is unknown. Suggested contributive factors in causing gastrointestinal cancers are; dietary-related (red meat intake\(^7\), folic acid\(^8\), fiber intake\(^9, 10\)), alcohol and smoking\(^11, 12\). Another associated factor is obvious but often overlooked and associated with the vast majority of solid tumors; age. Some macroscopic and microscopic precursor lesions of GI malignancies are known. In colorectal cancer sessile, serrated or hyperplastic polyps\(^13\) or aberrant crypt foc\(^14\) are recognized premalignant abnormalities and in gastric cancer intestinal metaplasia or foveolar hyperproliferation\(^15-17\). In premalignant lesions, cell division activity is disturbed, however, the cells remain within their histological architectural matrix. At some point, and this is the key step, the cells gain the capacity to invade through the basal membrane of the mucosa which defines the adenocarcinoma diagnosis. In the submucosal layer the cells can enter the lymphatic system or the bloodstream causing distant spread to lymph nodes or other organ sites causing GI cancer mortality.

<table>
<thead>
<tr>
<th>Cancer Type</th>
<th>Netherlands</th>
<th>United States</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>New cases (n in 2000)</td>
<td>Number of deaths (n in 2000)</td>
</tr>
<tr>
<td>Stomach</td>
<td>1938 (2134)</td>
<td>1450 (1719)</td>
</tr>
<tr>
<td>Colorectal</td>
<td>11231 (9236)</td>
<td>4709 (4274)</td>
</tr>
</tbody>
</table>

Table 1: GI cancer incidence and mortality rates in the Netherlands and United States in 2008.
This thesis aims at the two organs of the digestive system that are most often hit by malignant neoplasia: The stomach and the large bowel. Although over the last decades there has been an increase in the incidence of esophageal, gastric cardia and pancreatic cancer, this ranking remains unchanged over the last 20 years.

Gastric Cancer

Worldwide, neoplasms of the stomach (figure 1) are second in causing cancer mortality. Gastric cancer compared with other GI cancers is known for its geographically various incidence and mortality. In Asian countries such as China, Korea and Japan gastric cancer has the highest incidence and mortality of all malignancies. In Western countries approximately 50 years ago gastric cancer was also the most predominant cancer, although incidence rates dropped tremendously over the last decennia. In Portugal, however, gastric cancer incidence and mortality is highest and this is exceptional compared with other European countries. Dietary factors such as high intake of salted and smoked foods may explain this geographical variation. Increasing consumption of fresh ingredients in the wealthier Western world may account for the decline of gastric cancer incidence. Also the various incidences of gastric infections with Helicobacter Pylori bacteria or Epstein-Barr virus which are considered precursor stages of gastric cancer may contribute. Clinical symptoms are non-specific and mimic common diseases like dyspepsia or ulcer disease. Weight-loss or passage problems are late symptoms and the disease often is incurable at this stage. Five-year survival rates of gastric cancer in the Western world are deplorable (10-20%) and despite many efforts this has not markedly improved over the last years. Scientific interest for gastric cancer in Western countries has fainted as incidence rates drop. Gastric cancer’s relative unresponsiveness to the common systemic chemotherapeutics also contributes to this.
The mainstay of gastric cancer treatment is radical surgical removal of the complete or partial stomach with its surrounding lymph node stations. The extend of lymph node removal has been a subject of study and more extensive nodal dissection (including those surrounding the great vessels) may improve long-term disease survival rates, however, induces relatively high perioperative mortality in a Western study population. Recently, clinical trials have successfully shown the benefit of neoadjuvant therapy over surgery alone. These important studies now have generated more scientific interest to improve gastric cancer treatment. In the Netherlands the CRITICS trial is being conducted that aims to evaluate whether postoperative chemo/radiation therapy in addition to preoperative chemotherapy further improves survival rates.

**Colorectal Cancer**

The incidence of colorectal cancer in the Netherlands has increased with an average of one percent per year from 1989 to 2006 (www.ikcnet.nl) and forms a major burden on health care costs. It can be concluded that the treatment of colorectal cancer is improving as despite the increasing incidence, mortality rates have dropped. The large bowel appears anatomically and histologically as a continuous tube that is separated from the small bowel by Bauhin’s valve and ends at the dentate line where the squamous cells of the anal canal take over from the bowel mucosa epithelial cells. The last 15-20 centimeters distal of the promontorium or the sigmoid fold is called the rectum and this part of the colonic tube is fixed in the smaller pelvis between the bladder and the sacrum. The surgical technique for rectum resection is different from colon surgery due to this close interaction with the anatomical structures within the pelvic cavity. The fixation of the rectum provides opportunities for external beam radiation therapy. In literature large bowel cancer is often summarized as colorectal cancer while, as pointed out above, from a clinical point of view this should be separated into colon and rectal cancer.

**Colon Cancer**

From the cecum throughout the sigmoid the large bowel lies in the abdominal cavity and is hung up in the great omentum in the hepatic flexure and splenic flexure. Primary tumor removal can be achieved relatively simple in case there is no invasiveness into surrounding structures (T-stage 1-3). An important indicator of adequate surgery is to harvest sufficient amounts of lymph nodes for an adequate, complete pathological diagnostic procedure (pTNM-staging). Since nodal status is the most important clinical parameter to date much effort is put into optimizing nodal harvest and optimizing evaluation of the retrieved lymph nodes. Patients with nodal disease spread have shorter survival chances and have been proven to benefit from adjuvant chemotherapy. Different administration forms and schedules of the current standard FOLFOX-4 (5-FU, leucovorin, oxaliplatin) adjuvant treatment regimen are being studied to have added benefit. Addition of target drugs that specifically inhibit epidermal growth factor receptors (Cetuximab) or vascular endothelial growth factors inhibiting angiogenesis (Bevacuzimab or Avastin®) is under investigation. Yet 30 percent of patients without evidence of nodal spread will develop distant metastasis and this indicates the need for a better classification of this malignancy by prognostic markers.
Rectal Cancer

About 25% of adenocarcinoma in the 150 centimeters long large bowel occurs in the last 15 centimeters of the rectum (figure 2). The surgical treatment of rectal cancer, as indicated, is more challenging compared to colon cancer and can be complicated by specific morbidity. For instance accidental laceration of nerves of the sacral plexus can affect urinary and fecal continence and in males erectile function. To better protect the nerves and reduce perioperative blood loss Heald et al. in 1979 described total mesorectal excision (TME) meaning sharp dissection of the so-called avascular mesorectal fascia along with the rectum. TME has majorly reduced complications and local recurrence rates of rectal cancer. Many clinical trials have been conducted to further reduce local recurrence rates that test preoperative or postoperative strategies. Benefit of adjuvant therapy to reduce distant recurrence rates is currently under investigation in the SCRIPT trial (www.dccg.nl/trials/script). There are specific prognostic parameters in rectal cancer such as involvement of the circumferential margin and distance from the anal verge and these specific parameters should be included in studies evaluating rectal cancer.

Figure 2. Anatomy of the colon and rectum and its position in the abdomen.
The significance of the discovery of the molecular structure of DNA for clinical medicine has been gradually appreciated among physicians since 1953\(^{49-51}\). Molecular biology is a basic element of medical training nowadays. Medical students are taught the fundamental principles of DNA replication, its transcriptional regulation, the mechanism of protein synthesis and are made familiar with techniques for DNA analysis. Over the next years, molecular medicine will become increasingly integrated into daily medical practice. Oncology is an important field of research for molecular scientist where many new discoveries on disease mechanisms at the molecular level are being made. Cancer research and molecular oncology in particular will continue to be a field of invention as this disease still puzzles us and merits a larger portion of our scientific efforts.

Over the last years it has become increasingly clear that genetic alterations majorly contribute to the development of gastrointestinal adenocarcinoma. The nature (mutations, amplifications, translocations etc.) of genomic instability of GI cancer is being unraveled fast after the completion of the human genome project (www.genome.gov) and development of high resolution techniques that can analyze a individual’s complete genome in only a few weeks. On the DNA level, cancer cells differ greatly from healthy cells. Early in tumorigenesis genomic stability is affected and results into gene alterations that are key molecular pathogenic steps\(^{52}\). A current hypothesis is that by acquiring a sufficient number of alterations in tumor suppressor genes and oncogenes a normal bowel mucosa epithelial cell will transform and promote tumor progression\(^{53, 54}\). Identification of the key molecular changes of tumorigenesis will allow us to get a grasp on the malignant process and will offer targets for treatment. As our knowledge of cancer cell molecular biology expands, our models of disease mechanisms expand and certain dogmatic hypothesis need updating. Molecular medicine is an evolving field that is gaining importance in daily medical practice especially for those specialists involved in the treatment of cancer patients.

**Figure 3:** detail from the original publication presenting the helical structure of DNA by Watson and Crick and in Nature journal from 1953

**Molecular Oncology**

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Genetics versus Epigenetics

Alterations in the human genome involving the sequence of the four components of DNA, the nucleic acids (cytosine, guanine, adenine and thymine), are part of the field of genetics. Next to the nucleotides that form the double helix (Figure 4) there are many elements that are crucial for the main function of the human DNA, the process of protein transcription. These factors, i.e. methyl groups, histone proteins and their tails, chromatin structure remodeler complexes (Figure 4), are part of the world of epigenetics. The current definition of epigenetics is as follows: All heritable traits (over rounds of cell division, even trans-generationally) that do not involve the DNA nucleotide sequence. This new field is being more and more appreciated and also plays a key role in carcinogenesis as genetics does. As genetics is becoming part of our oncology world, epigenetic factors controlling transcription inevitably comes with it. This dissertation aims at one specific epigenetic factor namely, DNA methylation.
DNA methylation

The nucleus of each cell in the human body contains the same identical copy of genomic DNA. Differentiated cells from different organ structures perform specific functions requiring synthesis of specific proteins. Each cell, however, has the necessary information to produce all proteins encoded by the human genome. One can imagine that most cells have no need for the whole range of proteins. A large bowel mucosal cell for instance has no need for genes that encode proteins to form striated muscle. Synthesis of some proteins could disturb a cell’s homeostasis when produced. How embryonic cells develop into differentiated cells and how differentiated cells suppress transcription of unwanted or unnecessary proteins is an important question in cellular biology.

In 1948 it was first observed that a methyl-group was placed on the fifth position of the cytosine nucleotide (m5Cyt) in calf thymus DNA, 5 years before the molecular formation structure of DNA was established. Since then it has taken a dedicated group of scientists several decades to unravel what role this small methylgroup (CH₃) has in molecular cell biology. From the start, the hypothesis was postulated that cytosine methylation had a role in transcriptional regulation, however, there was no experimental evidence to support this. Waalwijk et al.’s classic experiments showed differential methylation patterns between various organ tissues in rabbits which was highly suggestive for the role of methylation in cellular differentiation. A role in differentiation was likely, as it was known that specific methylation patterns exist; that methylation was symmetrical in both DNA strands and that methylation patterns are clonally heritable. Other hypothesized functions of methylated DNA were that it would protect DNA from being cut by eukaryotic restriction enzymes. It also may play a role in DNA replication as was known that replication stops when DNA is unmethylated. A protective role was suggested as it was observed that unmethylated DNA is more prone to spontaneous mutagenesis. Also it was found that methylated sequences are more abundant in centromeric regions of a chromosome that indicates a role in chromosome structure and possibly folding.

Riggs et al. proposed in 1975 a model to explain the initiation and maintenance of mammalian X chromosome inactivation and certain aspects of other permanent events in eukaryotic cell differentiation. A key feature of the model is the proposal of sequence-specific DNA methylases that methylate unmethylated sites with great difficulty but easily methylate half-methylated sites. Peter Jones et al. in the late seventies made a key observation when he incubated mouse fibroblast-like embryonic cell lines with 5-azacytidine, a chemotherapeutic agent that was tested for treating leukemia. He noticed after several rounds of replication that the cells could be characterized as contractile striated muscle cells, differentiated adipocytes and chondrocytes capable of the biosynthesis of cartilage-specific proteins. He realized that this chemical must have activated genes that were silenced. Later on it was demonstrated that 5-aza was a specific inhibitor of DNA methyltransferase (DNMT) enzymes that are responsible for transferring methylation patterns from mother to daughter cell during replication.
Chemically, due to the open groove in the double helix, placement of a methyl-group is only possible when cytosine is followed by guanine. An important observation was that the CpG dinucleotide doublet is found less frequently in the human genome than is statistically expected. These doublets are more concentrated in coding regions compared with non-coding regions and this suggests they have a role in transcription. During the completion of “the human genome project” it could be more definitely supported that CpG dinucleotides are located predominantly in coding regions. More specifically, they are found in high concentration in gene promoter regions along with signaling molecules recognition sites (TATA boxes, helicase binding sites etc.) and those sites were named CpG islands. About 65% of all human genes have promoter regions that coincide with CpG islands. It is generally accepted that the human genome was more CpG rich earlier in evolution and that over time due to the earlier mentioned susceptibility of CG dinucleotides to spontaneous mutation/reduction to CT, only the CG’s protected by a methyl-group were conserved. Heavy methylation or hypermethylation of gene’s promoter region is associated with under-expression of that gene (see figure 5). This is likely due to the sterical inaccessibility of the promoter region. In short, the human genome contains CpG dinucleotide rich islands that coincide with gene promoter regions that are methylated or can undergo de novo methylation which results into silencing of that gene.

Figure 5: Representation of gene silencing by methylation. Boxed areas indicate gene exons.
- are methylated CpG dinucleotides,  are unmethylated CpG sites.
The arrows indicates the start site of transcription.

DNA methylation and cancer
Feinberg and Vogelstein were the first to test whether differences exist in DNA methylation between cancerous and non-cancerous cells of the same patient. They found that DNA of a variety of different human cancers was hypomethylated. They could show activation of some important oncogenes by hypomethylation, i.e. RAS, MAGE and CT. Subsequently, many genes thought to be important in cancer were studied for methylation status and surprisingly some genes were also found to be densely methylated (see table 2).
and this was first observed in calcitonin80. Later on, retinoblastoma (Rb), the first tumor suppressor gene, was found to be silenced by hypermethylation in cancer81, 82. A current hypothesis adds silencing of tumor related genes by DNA hypermethylation as another form of a second hit to Knudson’s hypothesis (see figure 6)83. One must look at methylation in cancer as an example of epigenetic dysregulation, with both hypomethylation and hypermethylation having significant roles.

<table>
<thead>
<tr>
<th>Gene abbreviation</th>
<th>Gene name</th>
<th>Location</th>
<th>Gene function</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
<td>5q21</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer 1</td>
<td>17q2</td>
<td>DNA repair</td>
</tr>
<tr>
<td>DAPK</td>
<td>Death associated protein kinase</td>
<td>9q34</td>
<td>Evasion of programmed cell death</td>
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<td>CDH1</td>
<td>E-cadherin</td>
<td>16q22.1</td>
<td>Cell adhesion</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
<td>6q25.1</td>
<td>DNA binding, transcription activation</td>
</tr>
<tr>
<td>GSTP1</td>
<td>Glutathione S-transferase P1</td>
<td>11q13</td>
<td>Cell cycle regulation</td>
</tr>
<tr>
<td>hMLH1</td>
<td>Human monologue of MutL in bacteria</td>
<td>3p21.3</td>
<td>DNA mismatches repair</td>
</tr>
<tr>
<td>MGMT</td>
<td>O-6- methylguanine-DNA methyltransferase</td>
<td>10q26</td>
<td>DNA repair</td>
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<td>RARb2</td>
<td>Retinoic acid receptor beta 2</td>
<td>3p24</td>
<td>DNA binding</td>
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<td>SOCS</td>
<td>Suppressor of cytokine signaling</td>
<td>17q25.3</td>
<td>Suppression of cytokine signaling</td>
</tr>
<tr>
<td>TIMP3</td>
<td>Tissue inhibitor of metalloproteinase 3</td>
<td>22q12</td>
<td>Tissue invasion and metastasis</td>
</tr>
</tbody>
</table>

_Tabel 2: Important tumor-related genes known to be silenced by promoter hypermethylation in solid tumors_ (source: Bernal et al., _Biol Res_41: 303-315, 2008)
Analytical Techniques
The unraveling of the role of DNA methylation in human biology and oncology has been hampered by technical limitations. Initially, chromatographic- (gas, liquid) or mass-spectrometry methods were used. The discovery of methylation-specific restriction enzymes was a major step forward, but analysis of methylation status of DNA sequences was limited to the recognition sites of the known enzymes. A major milestone in molecular research in general was the discovery of the protocol for polymerase chain reaction (PCR) by Kary B. Mullis described first in 1986. Any sequence of the human genome could now be amplified, with high specificity and reliability to a great amount of template majorly allowing rapid cloning and analysis of DNA. An important improvement in methylation research was the introduction of sodium metabisulfite treatment of DNA described by Suzanne Clark et al. first in 1994. The chemical instability of cytosine nucleotides and their proneness to deamination when unmethylated was utilized. DNA was incubated with a mild reductor (sodium metabisulfite) and showed specific conversion of cytosine nucleotides to thymine but not when methylated. Followed by direct sequencing analysis it can be detected if the DNA was methylated or not. Herman et al. then introduced DNA bisulfite treatment followed by PCR amplification with primers specific for methylated or unmethylated sequences. This protocol was highly sensitive and applicable in many laboratory settings and most importantly required only small amounts of sample DNA. When this thesis’ studies were initiated the most widely used technique for DNA methylation assessment was DNA bisulfite modification followed by methylation specific PCR (MSP) followed by gel electrophoresis of PCR products.

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**Figure 6:** Adjustment of Knudson’s hypothesis on gene inactivation

- **Normal**
- **TUMOR PROGRESSION**
- **Invasive cancer**

- **Active gene**
- **Partially active gene**
- **Inactive gene**

- **Promoter region methylation**
- **Gene mutation**
Micro Satellite Instability

In about fifteen percent of all cases of large bowel cancer there is positive family history. The most frequent form of hereditary colorectal cancer is Lynch Syndrome (former HNPCC). On the molecular level this cancer is hallmarked by mutations affecting DNA repair genes and thus impairing the proof reading mechanism after DNA replication in cell division. Mismatch repair (MMR) genes affected by mutations in Lynch Syndrome are MLH1, PMS2, MSH2 and MSH6. MMR deficiency can be detected by comparing the length of non-coding DNA repeat sequences, called microsatellites, of cancer cells with those of healthy cells that become shorter due to the defectiveness to repair these missing nucleotides after cell division. This so-called microsatellite instability (MSI) is also found in about 15% of colorectal patients without a positive family history and in whom no mutation in MMR genes is detected. Colorectal tumors with sporadic MSI show specific histologic features such as mucin excretion, poor differentiation, lymphocytic infiltration and clinical features such as location in the proximal colon and female sex. It has now been firmly established that in these patients shutdown of the hMLH1 mismatch repair gene by promoter region methylation is causative for sporadic MSI. This interesting interaction between genetics and epigenetics is further looked into in this thesis’s studies.

CpG Island Methylator Phenotype

The epigenetic silencing of tumor suppressor genes appealed to many researchers as a key causative mechanism of carcinogenesis and lead to a hunt for densely methylated CpG islands in human cancers. Issa et al. developed a screening method that compared human CRC cell line DNA to that of healthy donor lymphocytes. Differentially methylated sequences were located on the human genome, confirmed to adhere to the CpG island definition and this lead to 33 so-called methylated-in-tumor (MINT) loci. The MINT loci did not relate to any coding sequence or promoter region and some could be verified by methylation specific PCR to be specifically methylated in colorectal tumor tissue. It was observed that methylation of several MINT loci often occurred simultaneously and in concurrence with methylation of important tumor related genes such as p16 and hMLH1. Also, tumors with increased methylation showed MSI and a CpG island Methylator Phenotype (CIMP) colorectal cancer was proposed. Later, CIMP was further established as correlations were shown with KRAS, BRAF and p53 mutational status. The phenotype character of CIMP was challenged as this group of tumors could also constitute the far end of a continuous spectrum of methylation in tumor and not a distinct, clearly separable group. CIMP currently is still not clearly defined and different research groups use different marker-sets and techniques and show different clinical correlations. There are some consistent findings on clinical parameters associated with CIMP+ colorectal tumors: Older age, proximal location in colon, female sex, however, not surprisingly these all match MSI positive CRCs. Whether CIMP will be as significant in CRC as MSI is still under investigation.

Biomarkers

In daily medical practice, biomarkers are highly important tools for physicians in diagnosing diseases, estimating disease severity, follow-up of disease progression, exclusion of pathology etc. Next to diagnostics, markers are also used to help clinical decisions to start,
stop, adjust, switch or postpone treatment. Some biomarkers are direct reflectors of conditions (i.e. serum hemoglobin levels for anemia, however, indirect, surrogate markers are also frequently used that are reflective of conditions (i.e. C-reactive protein or CRP for infection). In GI cancer, the TNM-staging system is currently the best tool we have for making treatment decisions. There are indications, however, that there is room for improvement. First it is first important to realize that our treatment for GI cancer is empiric and non-specific. Aggressive surgery and chemo- and radiotherapy come with considerable morbidity and mortality. Better subclassification of GI tumors is needed to prevent undertreatment in some cases as well as overtreatment in others. The TNM system only includes surrogate parameters of disease progression. Primary tumor cell features, especially in GI cancers, currently have a limited role in clinical decision making. Molecular biomarkers assessed in the primary GI tumor form a new area of cancer diagnostics and are currently under investigation for their use. The use of epigenetic biomarkers in GI cancers is a relatively new field compared with genetic biomarkers. The single, most important objective of this thesis’ studies was to test whether epigenetic biomarkers have potential to subclassify GI cancer patients into clinically relevant groups. The role of this novel category of biomarkers in prediction of gastrointestinal cancer disease outcome was evaluated. Subsequently we continued to assess whether these markers merit further evaluation to make decisions tailoring the various multidisciplinary GI cancer treatment options for individual patients.
Reference List


General Introduction