Thymidylate Synthase Expression and Mismatch Repair Protein Expression in Colorectal Cancer

– Prognostic and Predictive Markers?

Katarina Öhrling
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Katarina Öhrling

Stockholm 2010
The cover picture is a MLH1 protein negative staining of an adenocarcinoma in the colon.

With dedication

to all patients who have
participated in these studies
and
To my family
ABSTRACT

The tumour, node, metastasis (TNM) system, with the current staging and risk stratification methods for prognostication in colorectal cancer (CRC) has its limitations. The need for additional validated prognostic and predictive markers is particularly important in CRC stage II and III as some of these patients can be cured by surgery alone.

In international tumour marker guidelines there is still not enough evidence to recommend the routine use of any tissue-based tumour markers in the treatment or surveillance of CRC. The problems are that the majority of studies evaluating tumour markers lack standardized methods for assessment, are non-randomized and involve a limited number of patients. Another main concern is the intrapatient heterogeneity of a certain tumour marker between the primary tumour and the corresponding metastases and even between different metastatic sites.

Two potential prognostic and predictive markers are:
1) Thymidylate synthase (TS), a rate-limiting enzyme involved in DNA synthesis and the target enzyme for 5-Fluorouracil (5-FU), which is the standard treatment used in CRC.
2) Microsatellite instability (MSI), the hallmark of a defective DNA mismatch repair (MMR) that occurs in about 15% of sporadic colorectal cancer (CRC).

In this thesis we have compared the expression of TS assessed with immunohistochemistry (IHC) in lymph node metastases as well as liver metastases and lung metastases of CRC with TS expression in matched primary tumours. Furthermore, we have evaluated the prognostic and predictive role of TS expression and MMR protein expression using IHC in stage II and III CRC.

A significant correlation was found between expression of TS in lymph node metastases and TS expression in their matched primary tumours. TS expression assessed in lymph node metastases did significantly improve the prognostic precision compared with TS expression in primary tumours, but did not predict response to 5-FU-based adjuvant chemotherapy. A low TS expression in lymph node metastases was associated with a longer OS and DFS.

TS expression was analyzed in liver metastases (n=38) and lung metastases (n=10) as well as in their matched primary tumours (n=45). There was no significant correlation between TS expression in distant metastases and their matched primary tumours. A tendency to a higher TS expression was seen in liver metastases (84%) compared to lung metastases (70%).

TS expression was a significant prognostic marker in patients treated with surgery alone where an improved survival was found in patients with low TS. Patients with the highest TS expression (grade 3) had a significantly improved survival when treated with adjuvant 5-FU-based chemotherapy independently of the dose of 5-FU.

MMR protein expression was found to be a significant prognostic marker for survival in univariate analysis as well as in multivariate analysis adjusted for gender, age, grade of differentiation, stage of disease and numbers of analyzed lymph nodes. Patients with MMR protein negative tumours had an improved survival compared to patients with MMR protein positive tumours. MMR protein expression did not predict benefit of adjuvant 5-FU-based chemotherapy with respect to survival.

In a combined analysis no significant correlation was revealed between MMR protein expression and TS expression in colon cancer. There was a significantly improved survival in stage III patients with MMR protein positive colon tumours expressing high TS when receiving adjuvant 5-FU-based chemotherapy compared to treatment with surgery alone.

It is questionable whether a single molecular marker may play a relevant prognostic and predictive role in a complex and heterogenous disease such as CRC. In the future we rather have to define different subtypes of CRC based on molecular, clinical and morphological features in order to tailor the optimal treatment for each individual patient maximizing the therapeutic effects whilst minimizing toxicity.

Key words: Sporadic CRC, Thymidylate synthase, Mismatch repair, Primary tumours, Metastases, Prognostic marker, Predictive marker.

LIST OF PUBLICATIONS

The thesis is based on the following publications, which will be referred to in the text by their Roman numerals:


Published articles have been reprinted with permission from Journal of Clinical Oncology, Anticancer Research and Acta Oncologica.
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>Avidin-biotin-peroxidase complex</td>
</tr>
<tr>
<td>ACF</td>
<td>A vacant crypt foci</td>
</tr>
<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>ASCO</td>
<td>American Society of Clinical Oncology</td>
</tr>
<tr>
<td>CH₂THF</td>
<td>5,10-methylene tetrahydrofolate</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
</tr>
<tr>
<td>CIMP</td>
<td>CpG island methylator phenotype</td>
</tr>
<tr>
<td>CIN</td>
<td>Chromosomal instability</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>CRM</td>
<td>Circumferential resection margin</td>
</tr>
<tr>
<td>CSS</td>
<td>Cancer specific survival</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DCC</td>
<td>Deleted in colorectal cancer</td>
</tr>
<tr>
<td>DFS</td>
<td>Disease-free survival</td>
</tr>
<tr>
<td>DPC</td>
<td>Deleted in pancreatic carcinoma</td>
</tr>
<tr>
<td>DPD</td>
<td>Dihydropyrimidine dehydrogenase</td>
</tr>
<tr>
<td>dUMP</td>
<td>Deoxyuridine monophosphate</td>
</tr>
<tr>
<td>dTMP</td>
<td>Deoxythymidine monophosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>Deoxythymidine triphosphate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ECOG</td>
<td>Eastern Cooperative Oncology Group</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGTM</td>
<td>European Group of Tumour Markers</td>
</tr>
<tr>
<td>ERCC1</td>
<td>Excision repair cross-complementing gene 1</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis coli</td>
</tr>
<tr>
<td>5dUMP</td>
<td>5-fluoro-2-deoxyuridine monophosphate</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Hereditary nonpolyposis colorectal cancer</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>LV</td>
<td>Leucovorin</td>
</tr>
<tr>
<td>MGMT</td>
<td>Methyl guanine methyltransferase</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch repair</td>
</tr>
<tr>
<td>MMRP</td>
<td>Mismatch repair protein</td>
</tr>
<tr>
<td>MSI</td>
<td>Microsatellite instability</td>
</tr>
<tr>
<td>MSI-H</td>
<td>Microsatellite instability high</td>
</tr>
<tr>
<td>MSI-L</td>
<td>Microsatellite instability low</td>
</tr>
<tr>
<td>MSS</td>
<td>Microsatellite stability</td>
</tr>
<tr>
<td>NSABP</td>
<td>National Surgical Adjuvant Breast and Bowel Project</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>PETACC</td>
<td>Pan-European Trials in Adjuvant Colon Cancer</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD-ECGF</td>
<td>Platelet derived endothelial cell growth factor</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression-free survival</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue deleted on chromosome ten</td>
</tr>
<tr>
<td>RTPCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SEER</td>
<td>Surveillance Epidemiology and End Results</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TILs</td>
<td>Tumour infiltrating lymphocytes</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue microarray</td>
</tr>
<tr>
<td>TME</td>
<td>Total mesorectal excision</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumour, node, metastasis</td>
</tr>
<tr>
<td>Topo-I</td>
<td>Topoisomerase-I</td>
</tr>
<tr>
<td>TP</td>
<td>Thymidine phosphorylase</td>
</tr>
<tr>
<td>TS</td>
<td>Thymidylate synthase</td>
</tr>
<tr>
<td>TSER</td>
<td>TS promoter enhancer region</td>
</tr>
<tr>
<td>UICC</td>
<td>International Union Against Cancer</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
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1 EPIDEMIOLOGY

1.1 Incidence and mortality

In terms of incidence colorectal cancer (CRC) is the fourth most common cancer in men and the third most common cancer in women worldwide [1]. Globally, there are about 1 million new cases of CRC and about half a million CRC deaths reported annually, according to data from the International Agency for Research on Cancer (IARC) [2].

The annual incidence of CRC in Sweden is approximately 62 cases per 100 000 inhabitants (65/100 000 males and 58/100 000 females) and CRC is the third most common cancer among men and the second most common cancer among women. The number of new CRC cases in Sweden in 2008 is listed in Table 1 [3]. The age-standardized mortality rates in CRC have gradually declined in Sweden since 1980, which might reflect improvements in CRC treatments that increase survival or an earlier detection due to a better recognition of symptoms [4]. Between 1960 and 1999 the 5-year survival rate in Sweden improved significantly in colon cancer from 39.6% to 57.2% and in rectal cancer from 36.1% to 57.6% [5].

<table>
<thead>
<tr>
<th>Tumoursite</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon</td>
<td>1874</td>
<td>1945</td>
<td>3819</td>
</tr>
<tr>
<td>Rectum</td>
<td>1104</td>
<td>762</td>
<td>1866</td>
</tr>
<tr>
<td>Total</td>
<td>2978</td>
<td>2707</td>
<td>5685</td>
</tr>
</tbody>
</table>

1.2 Hereditary factors

There are two major inherited syndromes of CRC, first syndromes with adenomatous polyps; hereditary nonpolyposis CRC (HNPCC) also called Lynch syndrome and familial adenomatous polyposis coli (FAP) and second syndromes comprising hamartomatous polyps; Peutz-Jeghers syndrome, Juvenile polyposis and Cowden syndrome. HNPCC accounts for 2-3%, FAP is found in < 1% and finally the rare hamartomatous polyposis syndromes represent < 0.1% of all CRC, (Figure 1) [6]. In addition there are individuals with a familial aggregation of CRC, where the specific genes are not yet identified that may account for 10-30%. Individuals with one first-degree relative with CRC double their lifetime risk, whereas individuals with two first-degree relatives have a threefold increased lifetime risk of developing CRC. The risk increases further with the numbers of relatives with an early onset of CRC (< 50 years) [7].
Figure 1. Distribution of sporadic-, familial- and hereditary colorectal cancer [6].
2 COLORECTAL CANCER DEVELOPMENT

2.1 Genetic pathways in colorectal cancer

Cancer development is a multistep process, where each step represents gene mutations or epigenetic changes in oncogenes, tumour suppressor genes or DNA-repair genes. When mutations accumulate the normal cell growth, change into a malignant behaviour with a capability to be self-sufficient characterised by loss of growth control, escape from apoptosis, sustained angiogenesis, invasion into surrounding tissue and metastatic spread [8]. CRC develop over years or decades via a number of distinct steps from a normal epithelium to a carcinoma described as the adenoma-carcinoma sequence [9]. Fearon and Vogelstein identified the genetic alterations involved in colorectal carcinogenesis and created a model for this multistep process, (Figure 2) [10].

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome location</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>5q21-22</td>
<td>Tumour suppressor gene</td>
</tr>
<tr>
<td>TGF-βR1</td>
<td>3p22</td>
<td>Cell signalling</td>
</tr>
<tr>
<td>MSH2</td>
<td>2p16</td>
<td>DNA mismatch repair</td>
</tr>
<tr>
<td>MLH1</td>
<td>3p21</td>
<td>DNA mismatch repair</td>
</tr>
<tr>
<td>MSH6</td>
<td>2p16</td>
<td>DNA mismatch repair</td>
</tr>
<tr>
<td>K-ras</td>
<td>12p12-1</td>
<td>Oncogene</td>
</tr>
<tr>
<td>p53</td>
<td>17p13</td>
<td>Tumour suppressor</td>
</tr>
<tr>
<td>Smad2/4</td>
<td>18q21-1</td>
<td>Tumour suppressor</td>
</tr>
<tr>
<td>p16INK4</td>
<td>9p21-3</td>
<td>Cell cycle control</td>
</tr>
<tr>
<td>COX2</td>
<td>1q25-2-3</td>
<td>Cell proliferation</td>
</tr>
<tr>
<td>DCC</td>
<td>18q21-3</td>
<td>Tumour suppressor gene</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>18q21-3</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>BAX</td>
<td>19q13-3-4</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>MGMT</td>
<td>10q26</td>
<td>DNA repair gene</td>
</tr>
<tr>
<td>PTEN</td>
<td>10q23</td>
<td>Tumour suppressor gene</td>
</tr>
</tbody>
</table>

Figure 2. APC, adenomatous polyposis coli; TGF-βR, transforming growth factor receptor; MSH, MutB homologue; MLH, MutL homologue; Smad, mothers against decapentaplegic homologue (Drosophila); COX, cyclooxygenase; DCC, deleted in colorectal cancer; Bcl, B-cell chronic lymphocytic leukaemia/lymphoma; BAX, Bcl-2-associated X protein; MGMT, O-6-methylguanine DNA methyltransferase; PTEN, phosphatase and tensin homologue [11]. Reprinted with permission Br J Surg 2006;93:395-406. Copyright 2006 © British Journal of Surgery Society Ltd. Published by John Wiley & Sons, Ltd.

The earliest genetic events are mutations in the tumour suppressor gene A denomatous Polyposis Coli (APC) on chromosome 5q21, which are found in 60–80% of sporadic colorectal cancers [12]. The autosomal dominant condition FAP is caused by germline mutations of the APC gene [13]. The APC gene product is a 312 kD multifunctional
protein localized in both the cytoplasm and the nucleus. APC may act as a negative regulator of \( \beta \)-catenin signaling in the transformation of colonic epithelial cells through the Wnt signaling pathway. Consequently, APC mutations result in an increased \( \beta \)-catenin activity in association with Tcf-4, a transcription factor [14]. One of the potential targets for this signaling is the CMYC gene, which is often upregulated in CRC [15]. Up to 40% of sporadic colorectal tumours have mutations of the KRAS gene [16]. The proteins found in the Ras family are involved in pathways of normal proliferation and differentiation, mediating signal transduction by surface receptors. The effect of KRAS mutations is not clear but seem to be diverse. An allelic loss on chromosome 18q occurs in approximately 70% of all CRCs and frequently also in adenomas [10]. A candidate gene from this region was identified already in 1990 as DCC (deleted in CRC), but the importance of DCC has been a subject of debate. However, recent observations suggest that DCC triggers cell death and is a receptor for netrin-1, a molecule implicated in colorectal tumorigenesis [17]. Furthermore, 18q contains a number of other tumour suppressor genes that seem to be important such as SMAD2 and SMAD4/DPC (Deleted in pancreatic carcinoma) [18,19]. Both SMAD2 and SMAD4 are involved in the TGF-\( \beta \) (Transforming growth factor beta) signaling pathway and mutations in these genes have been found in CRC. TP53 has a central role in many apoptotic pathways. Mutations in TP53 exist in up to 70% of all sporadic CRCs and lead to a transition from a benign adenoma to a malignant carcinoma that seem to occur before metastasis [20,21]. DNA damage in cycling cells stabilizes TP53 which activates other genes such as p21. This causes cell cycle arrest in the G1-phase and the DNA damage can be repaired before the cell cycle proceeds to the S-phase. When extensive DNA damage occurs TP53 instead activates pro-apoptotic genes for example the BAX gene which results in apoptosis [22].

Two different levels of genetic instability are involved in CRC development; chromosomal instability (CIN) and microsatellite instability (MSI). The majority (85%) of sporadic CRCs display CIN, while about 15% show MSI [23]. These two forms of genetic instability are defined as mutually exclusive, so that CRCs with CIN will display MSS (microsatellite stability) [24]. However, recent molecular biology studies indicate that the development of CRC not necessarily is divided into only two genetic pathways. In fact there is accumulating evidence of other routes of CRC carcinogenesis such as the serrated pathway and epigenetic instability as well as cross talk among different pathways [25]. The interplay of the various types of genomic and epigenomic instability is not clear and has to be further evaluated [26].
Chromosomal instability (CIN)

The chromosomal instability (CIN) phenotype exhibits gross chromosomal abnormalities such as aneuploidy and loss of heterozygosity (LOH). APC gene mutations are the most common initial molecular lesions in the CIN phenotype [27]. The APC protein performs a bridging function between microtubules and chromosomes during mitosis and a mutated APC gene results in defects in the segregation of chromosomes. CIN tumours are also characterized with other genetic alterations such as mutations in KRAS, SMAD2/4, TP53, and PI3KCA. The TGF-β signalling pathway seems to be important in the development of CRCs. In MSI tumours there is usually a mutation in the gene coding for the TGF-β receptor (TGFBR2), whereas in CIN tumours mutations of SMAD2/4 which lie further downstream in the same pathway is selected. The PI3KCA gene controls signaling pathways involved in cell proliferation, apoptosis, cell motility and cell invasion. Mutations in the PI3KCA have been found in about 30% of CRCs and seem to arise late in tumourigenesis [28].

Microsatellite instability (MSI)

Microsatellites are short repetitive DNA sequences (1-5 base pairs repeated 15–30 times), that are common throughout the genome [29]. The mismatch repair (MMR) system controls the repair of DNA base pair mismatches and is important for maintaining the genomic stability. There are at least six genes involved in the eukaryotic MMR system; MLH1, MSH2, PMS1, PMS2, MSH3 and MSH6 [30]. MSI is characterized by defects in the mismatch repair (MMR) system which leads to a size variation of microsatellites in tumour DNA compared to normal DNA. MSI was first described in sporadic CRC, but was later also identified in the autosomal dominant inherited syndrome, HNPCC [31]. In most sporadic CRC, an epigenetic change with hypermethylation of the MLH1 gene promoter results in its transcriptional silencing more than real mutations [32]. Germline mutations in the MMR genes, most commonly MLH1 (50%), MSH2 (39%) and MSH6 (7%) are the genetic basis for HNPCC. To date more than 400 different germline mutations of the MMR genes from over 700 families have been registered in the HNPCC database [33]. MSI is classified as either high (MSI-H) or low (MSI-L), depending on the extent of instability. Nearly all HNPCC-associated tumours display a MSI-H phenotype. Distinct molecular phenotypes have been found to distinguish MSI-H tumours from MSI-L tumours and tumours with MSS (microsatellite stability) [34]. The MSI tumours are almost always diploid and display only a few chromosomal aberrations [23]. Loss of MMR results in an increase in the mutation rate and MSI.
tumours are said to have a mutator phenotype [35]. It is suggested that the loss of MMR in sporadic CRC does not occur until after the usual steps of mutation in APC and KRAS [36]. BRAF mutations occur in about 10 to 18% of CRC overall and in 30 to 45% of MSI CRC, more frequently in tumours harbouring MLH1 promoter hypermethylation, whereas these mutations are not found in HNPCC-associated tumours [37,38]. Mutations in BRAF in MSI tumours might occur in the place of KRAS mutations, which are more common in CIN tumours [39]. After the loss of the MMR function the cells accumulate mutations of different genes especially in microsatellites and other nucleotide repeat sequences. Loss of function mutations have been found in genes coding for Type II TGF-β receptor (TGFBR2), the IGF II receptor (IGFIIIR), the BAX protein (BAX), the E2F4 cell regulator (E2F4) and the phosphatase and tensin homologue deleted on chromosome ten (PTEN) [40-44]. TP53 mutations occur in MSI tumours, but in a lower frequency than in CIN tumours. Instead mutations in the BAX gene, which is induced by TP53 as an effector of apoptosis, are common in MSI tumours.

The serrated pathway

Serrated adenomas, that represent 1–2% of colonic polyps combine architectural features of hyperplastic polyps and cytological features of classical adenomas and were first described in the 1990s [45]. The hypothesis of a third pathway to CRC, “the serrated pathway” is supported by the histological transition between hyperplastic aberrant crypt foci (ACF), hyperplastic polyps, mixed polyps, serrated adenomas and finally CRC [46]. Molecular studies comparing serrated adenoma and classical adenomas suggest that each may be a distinct entity. It has been demonstrated that 30–50% of serrated adenomas display MSI, mostly at a low level (MSI-L), and show a low rate of mutations in genes linked to the CIN-pathway such as APC, KRAS and TP53 [47]. In MSI-L serrated adenomas expression of the DNA-repair gene O-6-methylguanine methyltransferase (MGMT) seems to be lost by methylation of its promoter region [48]. However, among serrated adenomas with the MSI-H phenotype, an aberrant methylation CIMP (CpG island methylator phenotype) of the MLH1 gene with loss of its expression is frequently found. Serrated adenoma with MSI-H also have mutations in the same target genes as MSI-H cancer such as TGFBR2, IGFIIIR and BAX suggesting serrated adenomas as precursors to sporadic CRC with MSI [49].

Epigenomic instability

Epigenetic mechanisms play an important role in colorectal carcinogenesis such as decreased methylation defined as global hypomethylation or hypermethylation
described as the CIMP (CpG island methylator phenotype) [50]. Decreased methylation or global genomic hypomethylation is linked to CIN while CIMP is associated with MSI-H in patients without germline mutations in the MMR-genes. BRAF mutations are strongly linked to sporadic MSI tumours with CIMP-high (CIMP-H/CIMP P1) and these patients have a favourable prognosis [51]. A nother group of patients with a CIMP phenotype but mutations in KRAS are defined as CIMP-low (CIMP-L/CIMP P2) with lower levels of methylation [52]. In contrary to MSI CRC, increased levels of CIMP in MSS CRC seem to be associated with a worse prognosis [53].

2.2 Molecular classification of colorectal cancer

CRC is often viewed as a homogenous entity instead of a complex heterogeneous disease that develops through different genetic pathways. With focus on the form of genetic instability and the presence or absence of DNA methylation the pathologist Jass has categorized five molecular subtypes of CRC, (Table 2) [24]. However, additional genetic alterations such as mutations in APC, KRAS, BRAF, P53 are less specific and there are considerable overlaps between the different subtypes so this simplification has to be viewed with caution.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>MMR-status</th>
<th>CIMP-status</th>
<th>Ploidy</th>
<th>APC</th>
<th>KRAS</th>
<th>BRAF</th>
<th>P53</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (MSI)</td>
<td>MSI-H</td>
<td>CIMP-H</td>
<td>Diploid</td>
<td>wt/mut</td>
<td>wt</td>
<td>mut</td>
<td>wt</td>
<td>12%</td>
</tr>
<tr>
<td>2 (?)</td>
<td>MSS/MSI-L</td>
<td>CIMP-H</td>
<td>Diploid</td>
<td>wt/mut</td>
<td>wt/mut</td>
<td>mut</td>
<td>wt/mut</td>
<td>8%</td>
</tr>
<tr>
<td>3 (?)</td>
<td>MSS/MSI-L</td>
<td>CIMP-L</td>
<td>Aneuploid</td>
<td>mut</td>
<td>mut</td>
<td>wt</td>
<td>mut</td>
<td>20%</td>
</tr>
<tr>
<td>4 (CIN)</td>
<td>MSS</td>
<td>CIMP-neg</td>
<td>Aneuploid</td>
<td>mut</td>
<td>mut</td>
<td>wt</td>
<td>mut</td>
<td>57%</td>
</tr>
<tr>
<td>5 (HNPCC)</td>
<td>MSI-H</td>
<td>CIMP-neg</td>
<td>Diploid</td>
<td>mut</td>
<td>mut</td>
<td>wt</td>
<td>wt/mut</td>
<td>3%</td>
</tr>
</tbody>
</table>

MMR;Mismatch repair, MSI-H;Microsatellite instability high, MSI-L;Microsatellite instability low, MSS;Microsatellite stability, CIMP-H;CpG island methylator phenotype high, CIMP-L;CpG island methylator phenotype low, wt;wild type, mut;mutated.

2.3 Histological pathways of colorectal cancer

Over the last decade it has been suggested that an aberrant crypt foci (ACF), which consists of clusters of crypts where the epithelium is either dysplastic or hyperplastic, gives rise to an adenoma [54]. There are variations in the histological classification of ACF, where one approach is to classify ACF as either dysplastic or non-dysplastic [55]. Dysplastic ACF (microadenoma) progress to adenomas mostly growing as
polyps, while non-dysplastic ACF have been described as precursors for hyperplastic polyps [56]. Hyperplastic polyps are the most common type of polyps in colon and rectum and are rarely seen in patients before age 40 [57]. Traditionally hyperplastic polyps have been considered as innocuous lesions with no potential for progression to malignancy, but more recent reports have indicated that some subsets may have a malignant potential [58]. Histologically, sporadic ACF are mostly hyperplastic while the majority of FAP-associated ACF are dysplastic [56]. It has been suggested that ACF arise as a result of clonal genetic alterations. Gene mutations in for example APC and KRAS have been found in the ACF stage, although mutations in DCC and TP53 are more strictly displayed in the late stages of carcinogenesis. In 100% of dysplastic FAP ACF somatic APC mutations have been described compared to only 5% of the sporadic dysplastic ACF. In sporadic ACF KRAS mutations are detected in 63% of the dysplastic and in 82% of the non-dysplastic. In contrast, only 13% of the dysplastic FAP ACF showed KRAS mutations [59]. The MSI-phenotype seems to occur early in colorectal carcinogenesis as MSI is present in 100% of the ACF from patients with HNPCC and in 91% of the ACF from patients with sporadic MSI CRCs [60]. MSI is not associated with the histological features of ACF and is found in both dysplastic and hyperplastic ACF. Epigenetic alterations are common in CRC tumorigenesis and occur at early stages of ACF and polyp formations. DNA methylation has been demonstrated in 53% of sporadic ACF [61].
3 CLINICAL AND MORPHOLOGICAL FEATURES

It is not possible to solely categorize various subtypes of CRC on the basis of molecular findings. We also have to consider clinical and morphological features among different subtypes, (Table 3) [24]. However, there are clinical and morphological features that can be found in several molecular subtypes.

The clinical and morphological features are substantially different between CIN and MSI tumours. In summary, MSI tumours are more frequently right-sided, display an unusual histologic type (mucinous and medullary), are poorly differentiated, have a marked peritumoral and intratumoural lymphocytic infiltration [62].

Table 3. Clinical and morphological classification of colorectal cancer [24].

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Gender</th>
<th>Tumour site</th>
<th>Pre-cursor</th>
<th>Serration</th>
<th>Mucinous</th>
<th>Poor diff</th>
<th>Lymphocytic response</th>
<th>Circumscribed tumour border</th>
<th>Tumour budding</th>
<th>Dirty necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (MSI)</td>
<td>F&gt;M</td>
<td>R&gt;L</td>
<td>SP</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>2 (?)</td>
<td>F&gt;M</td>
<td>R&gt;L</td>
<td>SP</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>3 (?)</td>
<td>M&gt;F</td>
<td>L&gt;R</td>
<td>SP/AD</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>4 (CIN)</td>
<td>M&gt;F</td>
<td>L&gt;R</td>
<td>AD</td>
<td>+/−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>5 (HNPCC)</td>
<td>M&gt;F</td>
<td>R&gt;L</td>
<td>AD</td>
<td>+/−</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+/−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
</tbody>
</table>

MSI;Microsatelite instability, CIN;Chromosomal instability, HNPCC;Hereditary nonpolyposis colorectal cancer, F;Female, M;Male, R;Right, L;Left, SP;Serrated polyp, AD;Adenoma, diff;Differentiation.

3.1 Clinical features

Age and sex

CRC is a rare disease in young people and the median age at diagnosis of CRC in Sweden is 70−75 years. In Sweden colon cancer appears slightly more often in women while rectal cancer is a bit more common in men [3]. One study has demonstrated that sporadic MMR-deficient tumours develop at a higher age (mean 74.5 years) and are more commonly seen in women (68%) [63]. Another study reported that MSI-H tumours are diagnosed either before the age of 50 or over the age of 70 and predominantly in women [64]. Furthermore, an additional study revealed that MSI tumours are more common in women [65].

Tumour site

The proximal colon originates from the embryonic midgut, while the distal colon and rectum derive from the hindgut. Right-sided tumour are classified as originating
Clinical and morphological features

proximal to the splenic flexure (caecum, ascending colon, transverse colon), whereas left-sided tumours arise distally to this site (descending colon, sigmoid colon and rectum).

The anatomical distribution of colon and rectal cancer by site in percentages is shown in Figure 3 [66]. Historically, about 60% of CRC in high-incidence populations arise in the left colon, whereas there has been a predominance of right-sided CRC in low-incidence regions [67]. During the last decades there has been a shift towards proximal colon tumours in the Western countries, whereas the incidence of distal colon tumours has gradually decreased [68,69]. It has been demonstrated that right-sided colon cancer is frequently observed in elderly and female patients, whereas left-sided colon cancer is dominant in men and patients of middle age [70]. CRC patients with a family history of CRC are usually younger and their tumours are most often found in the right colon [71].

There are also distinct molecular differences between proximal and distal tumours. The DNA ploidy pattern seems to be site specific, where 80% of proximal tumours are diploid whereas only 40% of the distal tumours display diploidy [72].

Rectal cancer incorporates more mutations in comparison with colon cancer [73]. In the hereditary syndrome FAP, that is connected to the CIN pathway almost 100% of the individuals develop CRC in the left colon, whereas 70% of the patients with HNPCC with involvement in the MSI pathway are diagnosed with right-sided colon cancer [74]. In sporadic CRC, where 10-15% exhibits MSI there is site dependent difference in the presence of MSI with a higher frequency (30-35%) in proximal compared to distal tumours and it is very rare in rectal cancer [75,76]. However, the incidence of CIN is high in rectal cancer [77]. Somatic KRAS mutations seem to be equally distributed throughout the colon without site predominance, but rectal cancer has been described as less KRAS dependent [73,78]. Mutations in TP53 are most frequently seen in distal as well as rectal tumours [78,79]. Epigenetic changes such as hypermethylation described as the CIMP, which is closely related to the hypermethylation of the MLH1 gene in sporadic MSI tumours, are more often found in proximal tumours [80].

If the proximal and distal parts of the colon represent distinct entities, that might have potential implications in the therapeutic approach.
Pattern of metastasis

At the time of diagnosis up to 30% of the patients with CRC have metastatic disease [81]. The most common metastatic sites are the regional lymph nodes, liver, lungs and peritoneum [82]. Of all patients who die of advanced CRC 60-70% show liver metastases and metastatic spread to the liver is the main cause to CRC mortality [83]. Among CRC patients undergoing curative resection of the primary tumour 10-20% will develop hepatic metastases and 5-10% pulmonary metastases [84].

Metastatic spread in colon cancer is mainly lymphatic followed by a haematogenous route, with predominantly distant and/or peritoneal metastases but with a lower risk for local recurrences. The incidence of isolated lung metastases without liver metastases in colon cancer has been reported to range from 1-5.9% [85]. A recently published study based on colon cancer patient data collected from a German multicenter study revealed different anatomical sites for distant metastases, where peritoneal metastases were more frequently found with right-sided tumours, whereas hepatic and pulmonary metastases were more common in left-sided tumour [86]. In colon cancer an association between metastatic potential and M M R-status has been described where M S S tumours have a higher ability to develop metastases [87,88].

Local extension is predominant in rectal cancer with spread to the surrounding tissue and the risk for local recurrences is therefore high. There is a higher incidence of up to 12% of isolated lung metastases in rectal cancer compared to colon cancer and one potential explanation could be that the hematogenous spread in rectal cancer via the inferior and middle rectal veins can bypass the portal system [85].
3.2 Morphological features

Histological subtypes
The World Health Organization (WHO) has classified malignant primary tumours of the large intestine as epithelial tumours, carcinoid tumours and non-epithelial tumours [89]. The dominating histological type among epithelial tumours is adenocarcinoma, which accounts for 90–95%, where 10–20% has a mucinous component (> 50% of the tumour area is mucinous) and is defined as colloid or mucinous adenocarcinoma [65]. Medullary carcinoma, that was added to the revised WHO classification 2000 is a histological type strongly associated with a high degree of microsatellite instability (MSI-H) occurring either sporadically or in association with HNPCC and carries a more favourable prognosis [90]. In medullary carcinomas the malignant cells are large with abundant pink cytoplasm and vesicular nuclei with prominent nucleoli typically infiltrated with numerous lymphocytes in the malignant epithelium [91]. The signet ring cell carcinoma represents 2–4% of the mucinous carcinoma, where the cells contain high amounts of extracellular mucin pushing the nucleus to one side. More than 50% of the cells must demonstrate the “signet ring” in order to be classified as signet ring cell carcinoma, a histological type that carries an adverse prognosis and is applied to be poorly differentiated [92]. Less frequent histological types in the group of epithelial tumours include squamous carcinoma and undifferentiated carcinoma.

Grade of differentiation
The grading of adenocarcinomas is based on glandular formation. The majority of grading systems divide tumour into well differentiated, moderately differentiated, poorly differentiated and undifferentiated [93]. In clinical practice the histological grading has been questioned due to lack of a universally accepted criteria for determination of grade and has therefore had little impact on treatment decisions in the adjuvant setting [94]. However, a 2-tiered grading system, low grade (well differentiated and moderately differentiated) versus high grade (poorly differentiated and undifferentiated), has demonstrated a prognostic significance [95]. Several studies have reported that MSI-H carcinomas tend to be poorly differentiated with glandular formations in 5–50% of the tumour area [96,97].

Lymphocytic response
There are different patterns of lymphocytic response seen in CRC; peritumoural lymphocytes, Crohn like reaction, and tumour infiltrating lymphocytes (TILs) [63,98]. Peritumoural lymphocytes and a Crohn like reaction are more frequently found in HNPCC than in sporadic MSI-H CRCs [99].
TILs are closely related to MSI-H and medullary architecture but TILs have also been described in CRC with MSI-L and CIMP-high [100,101]. The lack of TILs have been reported as highly predictive of local recurrence in node negative MMR-competent CRC [102].

**Tumour border configuration and tumour budding**

The configuration of the invasive tumour border can be either infiltrating or expanding (pushing) and is a prognostic indicator in CRC. An infiltrating border characterized by an irregular, infiltrating pattern also known as tumour budding has mainly been described in MSS tumours [103]. Budding cells seem to have an increased sensitivity to mesenchymal growth signals and have been compared with malignant stem cells as they have a capacity to re-differentiate both locally and at the metastatic site [104]. In CRC tumours with MSI a well-delineated and circumscribed expanding (pushing) tumour border is the dominating tumour growth pattern, where the relative absence of tumour budding might explain their favourable prognosis [105, 106].

**Dirty necrosis**

CRC tumors can be assessed for the presence or absence of eosinophilic material within the glandular lumina, “dirty necrosis” using routine hematoxylin and eosin (H&E) stained sections. One study has demonstrated an inverse relationship between MSI-H tumours and the presence of dirty necrosis, where the lack of dirty necrosis was found to be an independent predictor of microsatellite instability [107].

**Venous and lymphatic vessel invasion**

Even if the results in the literature are conflicting there are evidence supporting the prognostic value of venous and lymphatic vessel invasion [100,108]. The problem is the lack of consensus in the method of examining and reporting vessel involvement.

**Perineural invasion**

Perineural invasion is the process of neoplastic invasion of nerves that is a somewhat underrecognized route of metastatic spread even if this pathologic feature is found in 20–30% of all CRCs at the time of surgery [109]. Studies have shown a significant correlation between perineural invasion in CRC and a higher risk of locoregional recurrence and metastatic disease as well as a worse clinical outcome [110]. The importance of perineural invasion has particularly been discussed in lymph node negative stage II CRC [111].
3.3 Staging

The pathological staging of CRC is complicated and various staging systems have evolved during the last century. In 1932, a Scottish pathologist named Dukes developed a classification for rectal cancer as stage A, B, or C that later also included colon cancer. Stage A was defined as tumour restricted to, but not through the bowel wall, stage B indicated penetration through the bowel wall and finally stage C described spread to local-regional lymph nodes, [112]. The Dukes’ staging system has thereafter been modified several times by adding new subgroups [113]. In 1954, Astler and Coller addressed the importance of depth of tumour penetration in patients with lymph node metastases changing C1 to node-positive with primary confined to the bowel wall and C2 was modified to node-positive with tumour penetrating the full thickness of bowel wall [114].

The tumour, node, metastasis (TNM) system classifying colorectal tumours due to invasiveness of the primary tumour (T), the number of lymph node metastases (N) and presence of distant metastases (M), was introduced in 1959 and has been gradually developed by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC). The TNM system is updated continuously based on new data on clinical outcome to improve prognostic information and guidance of adjuvant therapy. The most recent version of the TNM classification is the 7th edition of the AJCC Cancer Staging Manual effective for cancers diagnosed on or after January 1, 2010, that includes a revised substaging of stage II and III, (Table 4A) [115]. Interestingly, five new factors are recommended for routine assessment including; 1) satellite nodules, which means the number of peritumoral nodules or deposits of tumor that lack evidence of residual lymph node, 2) tumor regression grade for neoadjuvantly treated tumors, 3) the status of the circumferential resection margin (CRM), that is the surgical clearance in mm from the leading edge of tumor to the nearest dissected margin of the surgical resection, 4) MSI, an important prognostic and predictive factor for colon cancer; and 5) perineural invasion, which may be similar to lymph vascular invasion as an adverse prognostic factor.

A comparison of different pathological staging systems based on the 6th edition of the AJCC Cancer Staging Manual for CRC is shown in Table 4B [116]. In our studies we used the Dukes’ classification in study I-II and the 6th edition of the AJCC staging system in study III-V.
Clinical and morphological features

Table 4A. TNM classification according to the 7th edition of the AJCC Cancer Staging Manual [113].

<table>
<thead>
<tr>
<th>T (primary tumour)</th>
<th>N (nodal status)</th>
<th>M (distant metastases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>NX</td>
<td>MX</td>
</tr>
<tr>
<td>No evidence of primary tumour</td>
<td>No regional lymph node metastasis</td>
<td>No distant metastases</td>
</tr>
<tr>
<td>Tis (intraepithelial or intramucosal carcinoma)</td>
<td>N1a</td>
<td>M1a</td>
</tr>
<tr>
<td>T1</td>
<td>N1b</td>
<td>M1b</td>
</tr>
<tr>
<td>T2</td>
<td>N1c</td>
<td>M1b</td>
</tr>
<tr>
<td>T3</td>
<td>N2a</td>
<td>Metastasis in 4-6 regional lymph nodes</td>
</tr>
<tr>
<td></td>
<td>N2b</td>
<td>Metastasis in 7 or more regional lymph nodes</td>
</tr>
<tr>
<td>T4a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.4 Prognosis

One of the most important prognostic factors in the management of CRC is still lymph node status. To perform an adequate tumour staging in CRC the AJCC and the College of American Pathologist recommend at least 12 lymph nodes to be examined [116,117]. The largest study examining the impact of lymph node status to date is a review of the SEER (Surveillance, Epidemiology, and End Results) database, which demonstrated a 20% reduction in mortality when more than 15 lymph nodes were examined [118]. Sentinel lymph node mapping has been reported to be superior in identifying nodal involvement and could be one useful method to avoid understaging in the future [119].

The 5-year overall survival rates with surgery alone is correlated to the pathological stage at the time of diagnosis and range from 80-95% in stage I, 65-75% in stage II and 25-60% in stage III, (Table 4B). In stage IV the 5 year overall survival is less than 7% with chemotherapy alone but can increase to 10-15%, when metastasectomy after neodjuvant chemotherapy is attempted [120].

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>T1, N0, M0</td>
<td>A</td>
<td>A</td>
<td></td>
<td>80–95%</td>
</tr>
<tr>
<td></td>
<td>T2, N0, M0</td>
<td></td>
<td>B1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIa</td>
<td>T3, N0, M0</td>
<td>B</td>
<td>B2</td>
<td></td>
<td>72–75%</td>
</tr>
<tr>
<td>IIb</td>
<td>T4, N0, M0</td>
<td>B</td>
<td>B2</td>
<td></td>
<td>65–66%</td>
</tr>
<tr>
<td>IIIa</td>
<td>T1-2, N1, M0</td>
<td>C</td>
<td>C1</td>
<td></td>
<td>55–60%</td>
</tr>
<tr>
<td>IIIb</td>
<td>T3-4, N1, M0</td>
<td>C</td>
<td>C2, C3</td>
<td></td>
<td>35–42%</td>
</tr>
<tr>
<td>IIIc</td>
<td>Any T, N2, M0</td>
<td>C</td>
<td>C1, C2, C3</td>
<td></td>
<td>25–27%</td>
</tr>
<tr>
<td>IV</td>
<td>Any T, Any N, M1</td>
<td>D</td>
<td>D</td>
<td></td>
<td>0–7%</td>
</tr>
</tbody>
</table>
4 TREATMENT STRATEGIES

4.1 Surgery

Surgery is the primary treatment for CRC and over the past decades the ratio of CRC patients with a potentially curable disease has increased due to improved surgical techniques. The total mesorectal resection (TME) concept for rectal cancer, that was developed in 1982 and resulted in a considerable reduction of the local recurrence rates has become a standardized surgical technique [121].

The optimal approach to treatment of the primary tumour in patients with metastatic disease is a subject of debate as high mortality rates have been reported in prophylactic colon resection for this category of patients [122]. Surgical resection should mainly be offered to selected patients to prevent complications such as bowel obstruction or perforation and bleeding.

At the time of diagnosis approximately 15% of the patients with stage IV CRC have metastatic disease in the liver and less than 10% have pulmonary metastases that are resectable [123,124]. During the last 5 years, combination chemotherapeutic regimens integrating targeted monoclonal antibodies have allowed initially unresectable hepatic metastases to downsize and become resectable. The 5 year survival rates after resection of CRC liver metastases are 30–50%, but the risk of relapse is high [125]. After resection of pulmonary metastases 20–40% will survive after 5 years, but 70% will have recurrent disease [126]. A meta-analysis investigating adjuvant 5-FU after metastatic surgery in the liver showed a numerical but not significant improvement in DFS and OS [127]. The role of adjuvant chemotherapy after metastasectomy has to be further evaluated.

4.2 Adjuvant treatment

Colon cancer

Adjuvant chemotherapy has been developed to reduce the incidence of relapse and cancer-related death by eradicating micrometastases. Micrometastases are defined as small amounts of metastatic tumour that measure greater than 0.2 mm but less than 2.0 mm and should be classified as N1 if in a regional lymph node [116]. The role of adjuvant treatment is still a subject of debate in stage II colon cancer as approximately 75% of the patients are cured by surgery alone and adjuvant chemotherapy would only cure an additional 1–6% [128]. However, patients with stage III colon cancer are known to have a survival benefit of adjuvant chemotherapy around 30% [129].
5-Fluorouracil (5-FU) was developed in 1957 by Heidelberger and colleagues and still remains as the basic drug in the treatment with chemotherapy in CRC [130]. The main antitumoral effect of 5-FU is a competitive inhibition of thymidylate synthase (TS), a rate-limiting enzyme involved in DNA synthesis and repair. 5-FU can also exert its anticancer effects through incorporation of its metabolites into RNA and DNA, (Figure 4) [131]. Over the past 20 years, 5-FU biomodulation has led to the development of therapeutic strategies that increase its anticancer activity [132].

Figure 4. The metabolism of fluoropyrimidines [133]. Reproduced with permission from Eur J Cancer 2009 (45):1935-1949 Copyright 2009 © Elsevier Ltd.

5-FU was first combined with levamisole, an antihelminthic agent with immunomodulatory properties, where the potential antitumoral effects still remain unclear. 5-FU and levamisole for 12 months according to the Moertel scheme seemed promising in the treatment of stage III colon cancer compared with levamisole or surgery alone and was initially recommended as the standard adjuvant therapy in the United States and Europe [134].

The antitumor activity of 5-FU was subsequently shown to be enhanced when the drug was combined with leucovorin (LV), a chemically synthesized reduced folate, also referred to as folinic acid [135]. 5-FU and LV increased disease-free survival
Treatment strategies

(DFS) and overall survival (OS) in stage II and III colon cancer compared to 5-FU and levamisole [136]. It was also demonstrated that 6 months treatment with adjuvant 5-FU and LV was equivalent to 5-FU and LV administered for 12 months [137]. Furthermore, bolus and infusional 5-FU based schedules have similar effect in the adjuvant setting, but different toxicity profiles [138]. In Sweden, adjuvant chemotherapy for stage III colon cancer has been used since the mid 1990s where a Nordic schedule with bolus 5-FU and LV for 2 days every 2 weeks for 6 months is a standard [139].

In stage II colon cancer there is no single randomized clinical trial supporting adjuvant chemotherapy. However, the use of adjuvant chemotherapy is frequently discussed for stage II colon cancer patients with high-risk features such as T4 tumour stage, poor differentiation, venous and lymphatic vessel invasion and perineural invasion [128].

**Oral fluoropyrimidines, Irinotecan and Oxaliplatin**

Capecitabine is an oral prodrug of 5-FU, that has shown a similar efficacy as bolus 5-FU and LV in stage III colon cancer, (Figure 4) [140]. UFT, a combination of uracil that inhibits 5-FU degradation and tegafur, a pro-drug of 5-FU is also equivalent to bolus 5-FU and LV in terms of efficacy [141].

Irinotecan is a semisynthetic inhibitor of topoisomerase I, a nuclear enzyme important in DNA uncoiling for replication and transcription [142]. Although irinotecan is an established treatment in metastatic CRC both as a single agent and in combination with 5-FU and LV it has failed to prove any effect on clinical outcome in the adjuvant setting [143].

Oxaliplatin is a third-generation platinum compound that shows modest response rates as a single agent, but is very active in combination with 5-FU with evidence of a synergistic action. Oxaliplatin acts by producing DNA crosslinks of the DNA double helix that blocks DNA replication and transcription [144]. An international multicenter study (MOSAIC) demonstrated an improved DFS and OS in stage III colon cancer when 6 months infusional 5-FU was combined with oxaliplatin [145].

**Rectal cancer**

Adjuvant radiotherapy (preoperative or postoperative) decreases local recurrence rates by 50–60% compared to surgery alone and has become a standard procedure in the treatment of rectal cancer [146,147]. Over the last decades two different treatment schedules have been mainly used; a preoperative short term treatment where 25 Gy in 5 Gy fractions fractions are delivered during 5 days, immediately followed by surgery, and the more conventional long term postoperative schedule that delivers 40–50 Gy in 1.8 to 2 Gy fractions during 4–7 weeks [148-150]. In Sweden the short
A short term preoperative schedule with 5x5 Gy has become the standard treatment in resectable stage II and III rectal cancer. A randomized multicenter trial has also confirmed that the use of a short term preoperative schedule reduces the risk of local recurrence after optimized surgery using TME [151]. In locally advanced rectal cancer (T3-T4 tumours), the standard treatment is preoperative radiotherapy 50-50.4 Gy in 1.8-2 Gy fractions over 4–6 weeks combined with chemotherapy to achieve tumour down staging and increase the possibilities for a curative resection [152,153]. The attempts to define benefits of adjuvant chemotherapy in stage III rectal cancer have been more difficult as the evidence from randomized trials is very limited [154].

4.3 Treatment in metastatic colorectal cancer

During the last decade novel treatment modalities targeting (Epidermal growth factor receptor) EGFR- and vascular endothelial growth factor (VEGF) together with newer chemotherapeutic agents such as oxaliplatin and irinotecan have considerably improved the prognosis for patients with metastatic CRC. Due to the use of more efficient combination chemotherapeutic regimens a higher number of CRC patients with initially unresectable metastases will eventually become resectable. The advances in treatment of metastatic CRC have also had an impact on clinical outcome with an increased median overall survival from 6 months with best supportive care to more than 20 months in recent reports [155,156], Figure 5.

It has been suggested that 5-FU has a schedule-dependent mechanism of action, where bolus therapy results in an anti-RNA effect while infusional therapy has an anti-DNA effect [158]. In metastatic CRC, a meta-analysis has shown infusional 5-FU to be superior to 5-FU bolus in terms of tumour response and OS [159]. De Gramont et al. developed a regimen that combined two consecutive daily bolus 5-FU and LV with high-dose infusional 5-FU for 22 hours repeated every 14 days that demonstrated superior response rates compared to the daily x 5 Mayo bolus schedule and was found to have superior response rates [160]. Capecitabine as well as UFT seem to be equivalent with bolus 5-FU and LV in metastatic CRC [161,162]. A “doublet treatment” with a combination of 5-FU and LV with either oxaliplatin or irinotecan was proved to be more efficient than 5-FU and LV alone and were therefore established as standard treatments in metastatic disease [163,164]. Different “doublet regimens” have evolved based on a combination of bolus- plus infusional 5-FU together with either irinotecan (FOLFIRI) or oxaliplatin (FOLFOX), that seem to have equal efficacy independently of the sequence of administration of irinotecan or oxaliplatin but the toxicity profile differs [165].

A “triplet regimen” including both oxaliplatin and irinotecan in combination with 5-FU has also been tested, where a decrease in disease progression and an improved survival was found for FOLFOXIRI compared to FOLFIRI [155].

Cetuximab, a monoclonal antibody targeting EGFR has demonstrated effect both as a single drug and in combination with irinotecan [166]. Cetuximab also has a proven effect in combination with either FOLFIRI and FOLFOX in first line treatment [167,168]. The fully humanized antibody against EGFR, panitumumab, also seems to be effective in monotherapy as well as in combination with irinotecan or oxaliplatin in metastatic CRC [169-171]. The recently gained knowledge that the mutational status of the KRAS gene is predictive for the treatment effect of cetuximab and panitumumab have increased the possibilities to create an individualized therapy in metastatic CRC.

The addition of the humanized anti-VEGF monoclonal antibody, bevacizumab, to either FOLFIRI or FOLFOX in first line has revealed higher response rates but also an improved progression-free survival (PFS) [172,173]. Furthermore, bevacizumab also shows activity in second-line therapy together with FOLFOX [174]. The results from trials investigating the addition of an anti-EGFR antibody to a doublet regimen of chemotherapy plus anti-VEGF (bevacizumab) have so far been disappointing regarding response rates, PFS and OS [175,176].
5 PROGNOSTIC AND PREDICTIVE MARKERS IN COLORECTAL CANCER

The gold standard of prognostic markers in CRC is the UICC/AJCC TNM stage [115], but the current staging and risk stratification methods are insufficient especially in stage II and III disease as the stage variation in recurrences rates is wide reaching from 20-70% [177]. There is an urgent need for development of prognostic and predictive markers that focus on a personalized approach to the treatment of CRC, maximizing the therapeutic effects and minimizing toxicity.

When evaluating tumour markers that might be useful it is important to make a distinction between prognostic and predictive markers [178]. Prognostic markers correlate with clinical outcome independent of treatment, whereas predictive markers correlate with the impact of a specific treatment on outcome.

In recent years research on a global scale has tried to find prognostic and predictive markers in CRC with utility in clinical practice, where the success so far has been limited to the identification of wild-type KRAS as a predictor of response to anti-EGFR therapy in advanced CRC. Moreover, regular determination of the serum-based tumour marker CEA (carcinoma embryonic antigen) following curative surgery for CRC has been reported to be associated with outcome in two meta-analyses [179, 180]. In the ASCO (American Society of Oncology) 2006 as well as the European 2007 tumour marker guidelines it is now stated that CEA in postoperative surveillance should be analyzed every 2-3 months for at least 3 years for patients with stage II and III CRC [181,182]. However, it is still insufficient evidence to recommend the routine use of any tissue-based tumour marker that has been extensively evaluated during the last 5-10 years such as TS, MMR status, Loss of heterozygosity at 18q (18qLOH), TP53, dihydropyrimidine dehydrogenase (DPD) and thymidine phosphorylase (TP), in the treatment or surveillance of CRC [181,182]. The main reasons are conflict in published literature and lack of standardized methods for assessment and evaluation of potential tumour markers. Furthermore, most of the studies testing tumour markers have not included patients from randomized clinical trials and the number of patients in each study has usually been limited. Another problem that has been highlighted is whether analysis of a tumour marker should be performed on the primary tumour or on a metastatic lesion when predicting clinical outcome as well as response to chemotherapy in stage III and IV disease. It is also important to consider intrapatient heterogeneity of a certain tumour marker between the primary tumour and the corresponding metastases as well as between different metastatic sites as it might have potential implications in the therapeutic approach.

The main objective of the studies in this thesis is to evaluate the prognostic and predictive role of TS protein expression and MMR protein expression in CRC.
TS, a rate-limiting enzyme involved in DNA synthesis and the target enzyme for 5-FU [132], which is the standard treatment used in CRC, has been a tumour marker of interest for at least 15 years but is still not validated as a prognostic and predictive marker in CRC. However, the currently most consistent and convincing data are those related to MMR status involved in a form of genetic instability found in about 15% of the patients with sporadic CRC, which has been in focus of the research within this field for almost a decade [23].

It is important to address the changes made in the 7th edition of the AJCC Cancer Staging Manual, where AJCC recommends recording of the pre-operative level of CEA in every case and routine assessment of MSI as a new prognostic factor in colon cancer [115].

Chapter 5 gives a detailed background to the definition of TS expression and MMR protein expression as well as the available data in the literature addressing the role of these tumour markers to determine prognosis and predict response to chemotherapy in CRC. EGFR, 18qLOH, TP53, DPD, and TP are other potential prognostic and predictive markers of interest in CRC that will be more briefly summarized.

### 5.1 Thymidylate synthase (TS)

**Definition**

TS is a 36 kDa homodimeric protein which consists of two almost identical subunits and functions as a cytosolic enzyme [183]. The human TS gene is located in the telomeric region on the short arm of chromosome 18 at chromosome band 18p11.32 [184]. TS is the rate-limiting enzyme in the de novo synthesis of thymidine, one of the four nucleotides required for DNA synthesis and repair and is therefore an important target for chemotherapeutic agents, (Figure 6) [185]. TS catalyses the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), with the reduced folate 5,10-methylenetetrahydro-folate (CH₂THF) as the methyl donor. When dUMP and the reduced folate cofactor CH₂THF bind to the TS enzyme a stable ternary complex is formed. Continuous synthesis of dTMP requires regeneration of CH₂THF from dihydrofolate which occurs in two steps catalyzed by the enzymes dihydrofolate reductase and serine hydroxymethyltransferase. Once synthesized dTMP is further phosphorylated to deoxythymidine diphosphate (dTDP) and finally to deoxythymidine triphosphate (dTTP), which is a direct precursor of DNA-synthesis. There is also a “salvage pathway” where dTMP can be synthesized from thymidine by the cytosolic enzyme thymidine kinase 1 or the mitochondrial enzyme thymidine kinase 2 [186].
Regulation of TS expression

TS expression seems to be regulated by a highly polymorphic tandem repeat in the TS promoter enhancer region (TSER) from 2 to 9 copies of the 28 base pair sequence (2R to 9R), where double repeats (2R) or triple repeats (3R) are most common [188]. A greater in vitro enzyme activity has been reported with the triple repeat (3R) compared to the double repeat (2R) [189]. In patients with metastatic CRC homozygous for the double tandem repeats (TS 2R/2R) had a significantly lower TS expression than those with triple tandem repeats (TS 3R/3R) [190]. Interestingly, an ethnic variation in the TSER polymorphism has been reported among Caucasian and Asian populations [191]. In conclusion, these tandem repeats seem to affect the transcriptional as well as the translational efficiency of the TS gene.

TS levels vary throughout the cell cycle and an increase in TS enzyme activity occurs predominantly during S-phase [192]. Several clinical studies have shown that TS protein and TS mRNA levels are higher in different types of tumour cells than in normal cells and that high TS is associated with a poor clinical outcome [193-196]. It has been discussed whether TS overexpression is caused by an abnormal cellular proliferation or if TS overexpression in fact itself stimulates the development of a transformed phenotype with an increased cell growth and proliferation. E2F-1 is a protein that is involved in the transition from G1 to S-phase during cell cycle...
progression and has been identified as a potential regulator of the transcription of TS [197]. The activity of E2F-1 transcription factor has been reported to be under direct control of the retinoblastoma protein. When the retinoblastoma protein is inactivated E2F-1 is released that in turn activates the transcription of TS and other DNA synthesis enzymes [198]. Rahman et al. demonstrated behaviours of TS that are characteristic of an oncogene where an ectopic overexpression of TS transformed cells in vitro into a malignant phenotype [199]. If true it is important to combine agents that block the catalytic function of TS with therapies that also inhibit the transcriptional activity of E2F-1 or other targets along the retinoblastoma pathway.

**TS expression in different tumour lesions**

The primary tumour is often heterogeneous, where different parts of the primary tumour exhibit different TS activities [200]. Several reports have presented a significant discrepancy of TS expression between metastases and the corresponding primary tumours in CRC, (Table 11A-B) [201-206]. A few studies have also shown variable TS expression in different metastatic lesions from the same patient [203, 206-208]. One could therefore question the reliability of using analyses of TS expression or other tumour markers in the primary tumour to determine treatment for patients with metastatic CRC.

**TS inhibition**

Because TS is an essential enzyme for DNA-synthesis, which is also upregulated in malignant cells it has been viewed as a good target for various cytotoxic agents such as 5-FU, capecitabine, UFT, pemetrexed and ralitrexed.

**5-FU**

5-FU belongs to the class of antimetabolite drugs and inhibits TS after formation of the active nucleotide FdUMP. When FdUMP binds to TS it prevents the conversion of dUMP to dTMP leading to a “thymineless state”, causing DNA-damage that is cytotoxic to actively dividing cells, (Figure 4). The “salvage pathway” where thymidine can be converted to dTMP by the enzyme thymidine kinase can reverse this toxicity and represents a potential mechanism of resistance to 5-FU [132]. A variety of factors affect the ability of FdUMP to inhibit TS such as; the intracellular pool of FdUMP, the amount of the naturally occurring substrate dUMP, the enzyme activity of TS and finally the amount of reduced folate cofactor, CH$_2$THF [132]. The cofactor is essential for a tight binding of FdUMP to TS in order to form and maintain the ternary complex. The binding of FdUMP to TS is reversible in the absence of adequate amounts of cofactor. There is data supporting that the resistance
to 5-FU is dependent on the failure of FdUMP to create a stable ternary complex [209, 210]. 5-FU also has another mechanism of action and that is incorporation of the 5-FU metabolite FUTP directly into RNA, disrupting normal RNA processing and function [211].

**Capecitabine**

Capecitabine is an oral prodrug to 5-FU, that is absorbed as an intact substance and thereafter has to be activated by different enzymes, (Figure 6). The enzyme TP is responsible for the last step in this cascade and has been demonstrated with higher activity in malignant cells compared with normal cells. Capecitabine has therefore been described as tumour-selective [212].

**Other TS inhibitors**

DPD degrades 80% of the administered 5-FU to dihydrofluorouracil in the liver. Inhibition of DPD has been proposed as a strategy to increase the bioavailability of 5-FU. UFT is a combination of the DPD inhibitor uracil and the 5-FU prodrug, tegafur, that together with LV have demonstrated comparable efficacy with 5-FU and LV [141, 213]. Another available drug is eniluracil that combines UFT with LV [214].

Pemetrexed is a multi-targeted folate-based antimetabolite that inhibits various key folate-dependent enzymes but especially TS [215]. It is currently approved in combination with cisplatin for the treatment of non-small cell lung cancer (NSCLC) and malignant pleural mesothelioma but antitumour activity has also been found in CRC [216].

Ralitrexed is an analogue of the tetrahydrofolate cofactor (CH$_2$THF) and inhibits TS directly without requiring any modulating agent [217]. After transportation into the cell ralitrexed is polyglutamated, which increases the inhibition of TS but could also lead to increased toxicity. Early clinical trials showed similar activity to 5-FU, mainly in metastatic CRC, but with less grade 3 toxicity compared with 5-FU [218]. Further investigation of the drug was stopped due to greater treatment related mortality with ralitrexed in an adjuvant phase III trial [218]. Even if ralitrexed is still approved in many countries the use is mostly limited to patients with severe 5-FU toxicity.

**Methods of analysis**

There is a lack of a standardized assay for TS measurements. It has been reported, that TS mRNA levels correlate with TS protein levels determined by immunoblot and immunohistochemistry [193,219]. In the systematic review and meta-analysis by Popat et al. three different methods of analyzing TS were described; enzyme assays, immunohistochemistry (IHC) and reverse transcriptase polymerase chain reaction (RTPCR), where the most common used technique to determine TS was IHC [220].
Even if the majority of studies are based on TS analysis with IHC, different antibodies (monoclonal and polyclonal) as well as various methods of evaluation have been used. When analyzing gene polymorphism in the TSER with PCR it is not fully elucidated whether tumour tissue or normal tissue should be analyzed, even if most analysis so far have been based on tumour tissue [221].

**Immunohistochemistry (IHC)**

IHC is a semiquantitative method to detect TS protein levels using either a monoclonal or a polyclonal antibody in both fresh-frozen tumour tissue and formalin-fixed paraffin-embedded tumour blocks [222-224]. The advantages with IHC are the abilities to evaluate the histological features of the tumours and tumour heterogeneity. TS expression has most commonly been related to four chromagen intensity grades; (grade 0 = no staining, grade 1= weak staining, grade 2= moderate staining, grade 3= intense staining) [225]. Low intensity staining of TS has historically been defined as 0 and 1 and high intensity staining of TS as 2 and 3, a classification that was used in Study I-III [225-227]. In study III and V we have also added another categorization, where the intensity of TS expression is classified as low for TS grade 0-2 and as high for TS grade 3 [228]. Even if most reports have focused on the highest TS staining intensity found in the tumour some reports have instead quantified the proportion of stained cells per field using different arbitrary thresholds [229,230]. When analyzing the results from reports with IHC detected TS expression it is therefore also important to take into account the different methods of evaluation that have been used. In recent years IHC on tissue microarray (TMA) has been more frequently performed in tumour marker studies. The TMA technique is especially good at testing if genes found to be differentially expressed in CRCs by cDNA expression profiling are also distinct on the protein level [231].

**Reverse transcriptase polymerase chain reaction (RT-PCR)**

Quantitative gene expression data are often normalized to the expression levels of control or so-called “housekeeping” genes for example β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). An inherent assumption in the use of housekeeping genes is that expression of the genes remains constant in the cells or tissues under investigation. TS expression can be defined by quantifying the ratio of TS cDNA copy number amplified by RTPCR, to that of β-actin or GAPDH [232,233]. The amplification threshold used for quantization of TS gene expression varies between different studies and could be a potential source of bias [234,235].
TS as a prognostic marker and predictive marker

The impact of TS expression on clinical outcome as well as response to 5-FU-based chemotherapy depends on whether the analysis of TS has been performed on the primary colorectal tumour or on a metastatic lesion. In all adjuvant studies TS expression is tested on primary tumour, but in the advanced disease setting some studies assessed TS in distant metastases while other studies report TS expression solely from the primary tumour. When analyzing and comparing data from different studies it is therefore important to keep in mind which tissue origin that has been used to determine TS. As already mentioned, it is also essential to know that the results reported can be divergent depending on the method used to assess TS expression. Furthermore, recent studies suggest that TS gene polymorphism in the promoter enhancer region might be associated with clinical outcome and response to 5-FU [188].

TS as a prognostic marker

A meta-analysis evaluating TS expression and prognosis including both the adjuvant and advanced disease settings (n=3497), concluded that CRC tumors expressing high levels of TS have poorer OS compared with tumors expressing low levels [220]. In the adjuvant setting, where data from a total of 2610 patients were available for pooling, high TS expression only seemed to predict poorer OS for CRC patients treated by surgery alone. However, in all meta-analysis we have to be concerned with the possible publication bias as studies reporting significant findings are more likely to be published. Furthermore, in a recently published retrospective analysis of TS using TMA in the primary tumours from patients (n=556) with advanced CRC treated with capecitabine, irinotecan and oxaliplatin (the CAIRO study), TS did not have any prognostic value [133].

TS as a predictive marker

In patients with CRC treated adjuvantly with 5-FU and UFT a high TS expression in the primary tumor seem to be predictive for a better chemotherapy response, Table 5A [219,225,230,236-239, Study III]. Five studies have reported no significant difference in response to 5-FU with regards to the expression of TS [226-228,240,241]. Another report demonstrated a better response to neoadjuvant 5-FU in rectal tumours with a low TS expression [242]. A recent study with non-randomized stage II-III CRC patients treated with or without 5-FU-based chemotherapy using TMA and a polyclonal antibody, also found a better response to 5-FU in patients with a low TS grade in their tumours [243]. However, there is always a risk that the use of TMA underestimates the true frequency of high TS expressing tumours, particularly in
patients with focal staining. In summary, the results are conflicting and the potential use of TS as a predictor of benefit from adjuvant 5-FU still remains unclear.

Table 5A. TS expression as a predictive marker in adjuvant chemotherapy with 5-FU and UFT.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Ref.</th>
<th>No. of pts</th>
<th>Stage of disease</th>
<th>Lesion tested</th>
<th>Method</th>
<th>% High TS</th>
<th>Adjuvant treatment</th>
<th>Better response to adjuvant 5-FU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Johnston et al</td>
<td>1994</td>
<td>225</td>
<td>294</td>
<td>Dukes’A-D</td>
<td>Rectal ca</td>
<td>IHC(m)</td>
<td>69%</td>
<td>5-FU-based</td>
<td>High TS</td>
</tr>
<tr>
<td>Yamachicka et al</td>
<td>1998</td>
<td>230</td>
<td>86</td>
<td>Stage I-III</td>
<td>Colon ca</td>
<td>IHC(p)</td>
<td>19%</td>
<td>5-FU-based</td>
<td>High TS</td>
</tr>
<tr>
<td>Takenoue et al</td>
<td>2000</td>
<td>239</td>
<td>148</td>
<td>Stage I-IV</td>
<td>Colon ca</td>
<td>IHC(p)</td>
<td>28%</td>
<td>5-FU-based</td>
<td>High TS</td>
</tr>
<tr>
<td>Edler et al</td>
<td>2002</td>
<td>227</td>
<td>862</td>
<td>Stage II-III</td>
<td>CRC</td>
<td>IHC(m)</td>
<td>72%</td>
<td>5-FU-based</td>
<td>High TS=Low TS</td>
</tr>
<tr>
<td>Sugiyama et al</td>
<td>2002</td>
<td>238</td>
<td>245</td>
<td>Dukes’B+C</td>
<td>CRC</td>
<td>IHC(?)</td>
<td>36%</td>
<td>UFT</td>
<td>High TS</td>
</tr>
<tr>
<td>Allegra et al</td>
<td>2002</td>
<td>226</td>
<td>465</td>
<td>Dukes’B+C</td>
<td>Colon ca</td>
<td>IHC(m)</td>
<td>47%</td>
<td>5-FU-based</td>
<td>High TS=Low TS</td>
</tr>
<tr>
<td>Allegra et al</td>
<td>2003</td>
<td>228</td>
<td>706</td>
<td>Dukes’B+C</td>
<td>Colon ca</td>
<td>IHC(?)</td>
<td>40%</td>
<td>5-FU-based</td>
<td>High TS=Low TS</td>
</tr>
<tr>
<td>Kornmann et al</td>
<td>2003</td>
<td>219</td>
<td>309</td>
<td>Stage II-III</td>
<td>CRC</td>
<td>RTPCR+IHC</td>
<td>67%</td>
<td>5-FU-based</td>
<td>High TS</td>
</tr>
<tr>
<td>Jakob et al</td>
<td>2004</td>
<td>242</td>
<td>40</td>
<td>Stage II-III</td>
<td>Rectal ca</td>
<td>RTPCR</td>
<td>22%</td>
<td>5-FU-based</td>
<td>Low TS</td>
</tr>
<tr>
<td>Aguiar et al</td>
<td>2005</td>
<td>236</td>
<td>114</td>
<td>Stage II-III</td>
<td>CRC</td>
<td>IHC(m)</td>
<td>36%</td>
<td>5-FU-based</td>
<td>High TS</td>
</tr>
<tr>
<td>Westra et al</td>
<td>2005</td>
<td>241</td>
<td>391</td>
<td>Stage III</td>
<td>Colon ca</td>
<td>IHC(?)*</td>
<td>86%</td>
<td>5-FU-based</td>
<td>High TS=Low TS</td>
</tr>
<tr>
<td>Popat et al</td>
<td>2006</td>
<td>240</td>
<td>967</td>
<td>Stage I-III</td>
<td>CRC</td>
<td>IHC(?)</td>
<td>58%</td>
<td>5-FU-based</td>
<td>High TS=Low TS</td>
</tr>
<tr>
<td>Jensen et al</td>
<td>2007</td>
<td>237</td>
<td>303</td>
<td>Stage II-III</td>
<td>CRC</td>
<td>IHC(p)</td>
<td>24%</td>
<td>5-FU-based</td>
<td>High TS</td>
</tr>
<tr>
<td>Soong et al</td>
<td>2008</td>
<td>243</td>
<td>945</td>
<td>Stage II-III</td>
<td>CRC</td>
<td>IHC(?)*</td>
<td>39%</td>
<td>5-FU-based</td>
<td>Low TS</td>
</tr>
<tr>
<td>Study III</td>
<td>2010</td>
<td></td>
<td>1389</td>
<td>Stage II-III</td>
<td>CRC</td>
<td>IHC(m)</td>
<td>71%</td>
<td>5-FU-based</td>
<td>High TS</td>
</tr>
</tbody>
</table>

TS:Thymidylate synthase, 5-FU:5-Fluouracil, pts:patients, CRC:Colorectal cancer, IHC(m):immunohistochemistry monoclonal antibody, IHC(p):immunohistochemistry polyclonal antibody, IHC(?):immunohistochemistry antibody unknown, RTPCR:reverse transcriptase polymerase chain reaction, *,TMA:tissue microarray.

In metastatic CRC, a low TS expression in the metastases has been associated with a better response to 5-FU. Table 5B [203,229,233,235,244-250]. To date ten studies (n=830) have analysed the expression of TS in primary tumour as a predictor of response to chemotherapy with 5-FU in metastatic disease [232,251-259]. Six of these studies (n=333) demonstrated that a low TS in the primary tumour predicts a better response to 5-FU in metastatic CRC [232,251,254,257-259]. Interestingly, Aschele et al. found that a low TS was predictive for response to 5-FU when TS expression was assessed in the metastasis, but not in the corresponding primary tumour [201]. Whether chemosensitivity at metastatic sites can be predicted by TS expression in the primary tumour remains controversial. Almost all studies evaluating the predictive value of TS expression in metastases have been retrospective. A small prospective study (n=58) in patients with metastatic CRC, where 5-FU based chemotherapy was chosen based on a low expression of TS and DPD in distant metastases could not confirm a better response rate with low TS as reported in retrospective studies [260].
The ongoing ECOG study 4203 is a prospective study randomizing CRC patients with previously untreated metastatic or locally recurrent colorectal adenocarcinoma to different chemotherapeutic regimens based on TS expression. CRC patients with low TS expression will receive the FOLFOX regimen with bevacizumab, while patients with high TS expression are assigned to a combination of oxaliplatin, irinotecan and bevacizumab without 5-FU.
<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Ref.</th>
<th>No. of pts</th>
<th>Primary tumour</th>
<th>Metastatic site</th>
<th>Lesion tested</th>
<th>Method</th>
<th>% High TS</th>
<th>Treatment</th>
<th>Better response to S-FU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Findlay et al</td>
<td>1997</td>
<td>253</td>
<td>134</td>
<td>CRC</td>
<td>Local, Lung, Liver</td>
<td>Primary</td>
<td>IHC(p)</td>
<td>NE</td>
<td>5-FU-based</td>
<td>Low-TS=High-TS</td>
</tr>
<tr>
<td>Leichman et al</td>
<td>1997</td>
<td>248</td>
<td>42</td>
<td>CRC</td>
<td>Local, LN, Liver</td>
<td>Metastasis</td>
<td>RTPCR</td>
<td>50%</td>
<td>5-FU-based</td>
<td>Low TS</td>
</tr>
<tr>
<td>Kornmann et al</td>
<td>1997</td>
<td>247</td>
<td>29</td>
<td>CRC</td>
<td>Liver</td>
<td>Metastasis</td>
<td>RTPCR</td>
<td>38%</td>
<td>5-FU (HAI)</td>
<td>Low TS</td>
</tr>
<tr>
<td>Lenz et al</td>
<td>1998</td>
<td>233</td>
<td>36</td>
<td>CRC</td>
<td>NE</td>
<td>Metastasis</td>
<td>RTPCR</td>
<td>50%</td>
<td>5-FU-based</td>
<td>Low TS</td>
</tr>
<tr>
<td>Aschele et al</td>
<td>1999</td>
<td>245</td>
<td>48</td>
<td>CRC</td>
<td>Liver, Other</td>
<td>Metastasis</td>
<td>IHC(p)</td>
<td>44%</td>
<td>5-FU-based</td>
<td>Low TS</td>
</tr>
<tr>
<td>Bathe et al</td>
<td>1999</td>
<td>234</td>
<td>33</td>
<td>CRC</td>
<td>Liver</td>
<td>Metastasis</td>
<td>RTPCR</td>
<td>33%</td>
<td>5-FU-based</td>
<td>Low TS</td>
</tr>
<tr>
<td>Cascini et al</td>
<td>1999</td>
<td>203</td>
<td>41</td>
<td>Colon</td>
<td>Local, Liver</td>
<td>Metastasis</td>
<td>IHC(p)</td>
<td>66%</td>
<td>5-FU-based</td>
<td>Low TS</td>
</tr>
<tr>
<td>Davies et al</td>
<td>1999</td>
<td>246</td>
<td>36</td>
<td>CRC</td>
<td>Liver</td>
<td>Metastasis</td>
<td>IHC(m)</td>
<td>56%</td>
<td>5-FU(HAI)</td>
<td>Low TS</td>
</tr>
<tr>
<td>Aschele et al</td>
<td>2000</td>
<td>201</td>
<td>27</td>
<td>CRC</td>
<td>Local, LN, Liver</td>
<td>Metastasis</td>
<td>IHC(?)</td>
<td>48%</td>
<td>5-FU-based</td>
<td>Low TS</td>
</tr>
<tr>
<td>Aschele et al</td>
<td>2000</td>
<td>201</td>
<td>27</td>
<td>CRC</td>
<td>Local, LN, Liver</td>
<td>Metastasis</td>
<td>IHC(?)</td>
<td>70%</td>
<td>5-FU-based</td>
<td>Low-TS=High-TS</td>
</tr>
<tr>
<td>Paradiso et al</td>
<td>2000</td>
<td>258</td>
<td>108</td>
<td>CRC</td>
<td>Liver, Other</td>
<td>Primary</td>
<td>IHC(m)</td>
<td>50%</td>
<td>5-FU-based</td>
<td>Low TS</td>
</tr>
<tr>
<td>Salonga et al</td>
<td>2000</td>
<td>249</td>
<td>33</td>
<td>CRC</td>
<td>Local, LN, Liver</td>
<td>Metastasis</td>
<td>RTPCR</td>
<td>67%</td>
<td>5-FU-based</td>
<td>Low TS</td>
</tr>
<tr>
<td>Shirota et al</td>
<td>2001</td>
<td>250</td>
<td>50</td>
<td>CRC</td>
<td>Local, Liver</td>
<td>Metastasis</td>
<td>RTPCR</td>
<td>14%</td>
<td>5-FU-based</td>
<td>Low TS</td>
</tr>
<tr>
<td>Aschele et al</td>
<td>2002</td>
<td>244</td>
<td>124</td>
<td>CRC</td>
<td>Local, Lung, Liver</td>
<td>Metastasis</td>
<td>IHC(p)</td>
<td>52%</td>
<td>5-FU-based</td>
<td>Low TS</td>
</tr>
<tr>
<td>Berglund et al</td>
<td>2002</td>
<td>252</td>
<td>122</td>
<td>CRC</td>
<td>NE</td>
<td>Primary</td>
<td>IHC(m)</td>
<td>78%</td>
<td>5-FU-based</td>
<td>Low-TS=High-TS</td>
</tr>
<tr>
<td>Gonen et al</td>
<td>2003</td>
<td>229</td>
<td>156</td>
<td>CRC</td>
<td>Liver</td>
<td>Metastasis</td>
<td>IHC</td>
<td>15%</td>
<td>5-FU+(HAI)</td>
<td>Low TS</td>
</tr>
<tr>
<td>Farrugia et al</td>
<td>2003</td>
<td>235</td>
<td>20</td>
<td>CRC</td>
<td>NE</td>
<td>Metastasis</td>
<td>RTPCR+IHC(p)</td>
<td>62%/52%</td>
<td>5-FU-based</td>
<td>Low TS</td>
</tr>
<tr>
<td>Ichikawa et al</td>
<td>2003</td>
<td>232</td>
<td>37</td>
<td>CRC</td>
<td>LN, Lung, Liver</td>
<td>Primary</td>
<td>RTPCR</td>
<td>51%</td>
<td>UFT</td>
<td>Low TS</td>
</tr>
<tr>
<td>Johnston et al</td>
<td>2003</td>
<td>255</td>
<td>219</td>
<td>CRC</td>
<td>NE</td>
<td>Primary</td>
<td>IHC(m)</td>
<td>80%</td>
<td>5-FU-based</td>
<td>Low-TS=High-TS</td>
</tr>
<tr>
<td>Bendardaf et al</td>
<td>2005</td>
<td>231</td>
<td>54</td>
<td>CRC</td>
<td>NE</td>
<td>Primary</td>
<td>IHC</td>
<td>48%</td>
<td>5-FU-based</td>
<td>Low TS</td>
</tr>
<tr>
<td>Matsuyama et al</td>
<td>2006</td>
<td>256</td>
<td>22</td>
<td>CRC</td>
<td>Liver</td>
<td>Primary</td>
<td>RTPCR</td>
<td>NE</td>
<td>5-FU (HAI)</td>
<td>Low-TS=High-TS</td>
</tr>
<tr>
<td>Iyeleva et al</td>
<td>2007</td>
<td>254</td>
<td>29</td>
<td>CRC</td>
<td>NE</td>
<td>Primary</td>
<td>RTPCR</td>
<td>45%</td>
<td>5-FU-based</td>
<td>Low TS</td>
</tr>
<tr>
<td>Yanagisawa et al</td>
<td>2007</td>
<td>259</td>
<td>13</td>
<td>CRC</td>
<td>LN, Lung, Liver, Other</td>
<td>Primary</td>
<td>RTPCR</td>
<td>38%</td>
<td>5-FU-based</td>
<td>Low TS</td>
</tr>
<tr>
<td>Nakajima et al</td>
<td>2008</td>
<td>257</td>
<td>92</td>
<td>CRC</td>
<td>LN, Lung, Liver, Other</td>
<td>Primary</td>
<td>RTPCR</td>
<td>30%</td>
<td>5-FU-based</td>
<td>Low TS</td>
</tr>
</tbody>
</table>

TS; Thymidylate synthase, 5-FU; 5-Fluorouracil, pts; patients, CRC; Colorectal cancer, LN; lymph nodes, NE; not evaluated, IHC(m); immunohistochemistry monoclonal antibody, IHC(p); immunohistochemistry polyclonal antibody, IHC(?); immunohistochemistry antibody unknown, RTPCR; reverse transcriptase polymerase chain reaction, *; TMA; tissue microarray, HAI; hepatic artery infusion.
5.2 Mismatch repair (MMR) and Microsatellite instability (MSI)

Definition

The cell needs different types of DNA repair mechanisms, where damaged DNA can be recognized and removed. DNA damage may be either limited to altered DNA bases or extensive like double-strand breaks. If DNA repair is unsuccessful this may lead to permanent cell cycle arrest (cellular senescence), apoptosis, or oncogenesis. Four types of DNA repair systems have been described such as base excision repair, nucleotide excision repair, double strand break repair, and MMR [261]. The MMR system recognizes and repairs errors caused by the regular DNA replication machinery for example single mismatched base pairs or small insertions or deletions of nucleotides. (Figure 7). The MMR process begins when the MSH2-MSH6 protein complex also defined as MutSα recognizes and binds to single mismatched base pairs [262]. Another protein complex MSH2-MSH3 (MutSβ) is responsible for the repair of larger (2-4 base pairs) mismatches. Thereafter, two other proteins, MLH1 and PMS2 described as MutLα are recruited to the complex for activation and initiation of the repair events. After the mismatched strands have been cleaved, an exonuclease removes the segment from the cleavage site and finally DNA polymerase-α fills in the single-strand gap. The link between MSI and cancer was first described in 1993 [31]. Microsatellites are as already mentioned in Chapter 2 short repetitive DNA sequences (1-5 base pairs repeated 15-30 times), that are common throughout the genome [29]. DNA replication errors can lead to single nucleotide mutations and length alterations in these microsatellites and if they accumulate that can result in MSI. Defects in four proteins of the MMR system (MLH1, MSH2, MSH6 and PMS2) have been described in CRC and are associated with MSI. In HNPCC MSI is a result from germline mutations in the MMR genes, where mutations in MLH1 and MSH2 account for more than 90% [33]. In about 15% of the sporadic CRC cases hypermethylation of the MMR gene promoter, most commonly MLH1, leads to a defective MMR [32].

MMR defective tumours have distinctive clinical and morphological features as already described in Chapter 3.
**Figure 7.** The mismatch repair system [263]. Reprinted with permission from Cancer Biomark. 2006;2(1-2):51-60. Copyright 2006 © IOS Press and the authors, All rights reserved.

### MMR in different tumour lesions

The incidence of MSI in sporadic primary colorectal tumours is about 15% [264]. Data on the incidence of MSI in primary tumours of patients with stage IV CRC are limited, but the most recent studies have reported an incidence between 3.5-8% [87, 265-267]. A lower risk of developing metastases have been found in CRC patients with MSI tumours and this could perhaps explain the lower frequency of MSI in a selected group of stage IV patients [87,88,268]. The two largest studies to date evaluating MSI in liver metastases each with more than a hundred stage IV patients, who underwent hepatic resection reported frequencies of MSI of 2.5 and 2.7 % respectively [269, 270]. In a study including 56 stage IV patients with unresectable liver metastases they found MSI in the liver biopsy from only one patient, (1.8%) [271]. In a report with 81 patients who underwent pulmonary resection all lung metastases displayed MSS [272]. An early study comparing MSI in the primary tumour (n=10) with MSI in 56 corresponding metastases (lymph node n=26, liver n=1, omentum n=1) found no difference in MSI frequency between the different tumour sites [273]. This result is in accordance with a recent study in stage III CRC, where no significant difference in MSI frequency was found in 47 primary tumours and their corresponding lymph node metastases [274]. Furthermore, another report analyzing protein expression of MLH1, MSH2 and PMS2 in 92 primary tumours and
corresponding lymph node metastases and liver metastases found differential expression of MLH1/PMS2 between primary tumours and liver metastases [266].

Methods of analysis

PCR

PCR has been the most widely used technique and is considered as the “gold standard” to detect MSI. The tumour tissue can be fresh-frozen or formalin-fixed paraffin embedded. If the tumour-samples are paraffin-embedded a microdissection is required for an optimal tumour yield and to receive comparable normal cells from adjacent colon mucosa. In the 1997 international Bethesda guidelines a reference panel of five microsatellite markers was proposed for the analysis of MSI including two mononucleotide (BAT-25 and BAT-26) and three dinucleotide (D5S346, D2S123, and D17S250) repeats [264]. Tumours are classified as MSI-H if at least 2/5 markers display instability or more than 30% if more than five markers are used. If 1/5 markers or less than 30% display instability the tumour is classified as MSI-L. Finally, in microsatellite stable (MSS) tumours at least five markers were required to show stability. These guidelines were revised at a National Institutes of Health (NIH) conference in 2004, where an additional confirmation with mononucleotide markers was recommended if MSI had been solely defined with dinucleotide markers [275]. Overall, about 60-70% of CRC tumours are MSS, whereas 15-20% are MSI-L and 15-20% are MSI-H [264]. In most reports, MSI-L and MSS tumours are defined as one group in comparison with MSI-H tumours. However, there is a need for further evaluation of MSI-L as some investigators indicate that these tumours might represent a separate entity [276,277].

IHC

Several studies have shown that IHC assessment of MLH1 and MSH2 protein expression has a high degree of correlation with the MSI phenotype determined by PCR. The sensitivity to detect MLH1 and MSH2 defective tumours with IHC is 80-95% and the specificity has reached 100% in most reports [278-282]. Analysis of MLH1 and MSH2 protein expression using IHC has some clear advantages over MSI testing with PCR. IHC can easily at a lower cost define the MMR status in paraffin-embedded formalin fixed tissue that can be carried out even if only a small amount of tumour tissue is available for example colonoscopic biopsies. When using IHC it is also possible to evaluate histological features of the tumour and tumour heterogeneity. If HNPCC is under investigation MMR protein staining with IHC has the benefit of guiding the clinicians to the correct gene and thereby facilitate the mutation analysis. On the other hand, IHC will always be semi-quantitative and
therefore difficult to standardize. When MSI status is defined with IHC analysis of solely MLH1 and MSH2 protein expression two hits to any of the other components of the DNA MMR system can be missed for example MSH6 and PMS2. It is also important to be aware of the difficulties in technique and interpretation of MMR IHC assays [283]. Absence of or low intensity staining can be linked to the sensitivity of antigens to fixation time or the duration of the storage of the paraffin-embedded tumour slides [284,285]. In a study of sporadic CRC comparing IHC with PCR in detecting MSI, they concluded that IHC cannot replace PCR as long as the staining protocols are not optimized, as IHC failed to detect 36% of the cancers with MSI in their study cohort [286]. When there is a discrepancy in the results between IHC and PCR in detecting MSI tumours different clinical, biological as well as technical explanations have to be considered before claiming that one test is wrong [281].

**MMR as a prognostic and predictive marker**

It is hard to compare the results between studies addressing the prognostic and predictive role of MSI in CRC as various methods (PCR and IHC) have been used to determine MSI in different tumour locations (colon and rectum) and treatment settings (adjuvant and palliative). Furthermore, most of the studies have not included patients from randomized clinical trials and the number of patients in each study is usually small. The majority of the previous studies have used PCR to identify MSI even if IHC is becoming a more widely used technique.

**MMR as a prognostic marker**

The relationship between MSI status by genotyping and a better survival has been demonstrated in a systematic review including 32 primarily retrospective studies with 7642 CRC cases with both locally advanced (stage II and III) and advanced disease (stage IV), [287]. Thereafter, another five studies have shown a significantly better prognosis among patients with MMR deficient CRC, (Table 13) [87,96,288,289, Study IV]. The mechanisms, that contribute to a better prognosis in CRC tumours with MSI are not fully understood but might be related to an inflammatory response with functionally active lymphocytes [268,290].

**MMR as a predictive marker**

In vitro studies have shown that CRC cells with MSI are less responsive to 5-FU because of a tolerance to acquired DNA damage [291-293]. More specifically, components of the DNA mismatch repair system have been shown to recognize and bind to 5-FU that becomes incorporated into DNA, which could be a trigger to induce cell death. The binding and subsequent cell death events would be absent in
Prognostic and predictive markers in colorectal cancer

colorectal tumours with MSI, which have lost their DNA mismatch repair function. The use of hypomethylating agents in order to cause a re-expression of MLH1 and thereby convert resistant hypermethylated MLH1 deficient CRC tumours to become sensitive to 5-FU, has been discussed as a new treatment approach [294]. 5-FU in combination with oxaliplatin is the accepted standard adjuvant treatment of colon cancer stage III today and there is a need for molecular markers that can predict the treatment effect of this drug. The MMR status seems not to be involved in resistance to oxaliplatin, even if it is a potential resistance mechanism to other platinum drugs [295]. An increased sensitivity to topoisomerase inhibitors such as irinotecan has been demonstrated in MMR deficient colorectal cancer cell lines but the molecular mechanism behind this is still not clear [296].

Consistent with these preclinical findings, a randomized retrospective study reported a survival advantage in 5-FU treated CRC patients with MSI-L and MSS cancers, but not in patients with MSI-H tumours [297]. Two other non-randomized retrospective studies also found a benefit of 5-FU in patients without MSI [96, 298]. In a recent pooled molecular reanalysis of randomized chemotherapy trials, deficient mismatch-repair was found to be a predictive marker for lack of benefit from 5-FU based chemotherapy in stage II and III colon cancer [299]. In contrast with these findings early clinical trials indicated that 5-FU was beneficial for CRC patients with MSI tumours [300,301]. However, without randomization between patients treated with either adjuvant chemotherapy or surgery alone it is hard to evaluate whether the improved survival was due to the MMR-status in the tumours or the chemotherapy in itself. The first prospective study of the use of adjuvant 5-FU based on the MMR status also showed a better OS among CRC patients with MMR competent tumours treated adjuvantly with 5-FU-based chemotherapy [302,303]. A another retrospective study demonstrated that MSI did not correlate with benefit of 5-FU-based chemotherapy [304]. Furthermore, an additional retrospective study from 2007 with data from prior National Surgical Adjuvant Breast and Bowel Project (NSABP) Collaboration Studies does not support the use of MSI as a predictor of benefit of chemotherapy [305]. On the other hand, in this retrospective cross-study comparison where the patients treated with adjuvant chemotherapy and the untreated controls were collected from separate clinical trials there is always a high risk of selection bias. A systematic review with meta-analysis evaluating the ability of MSI to predict efficacy of adjuvant chemotherapy concludes that MSI-H status is a predictive factor of non-response in the adjuvant setting [306]. Studies that have described MMR status as a predictive marker in adjuvant 5-FU-based chemotherapy are listed in Table 6A [96,297-305, Study IV]. Furthermore, a recently published study showed that loss of MMR function may predict improved outcome in stage III colon cancer patients treated adjuvantly with irinotecan and 5-FU compared with those patients only receiving 5-FU [307].
Table 6A. MMR status as a predictive marker in adjuvant 5-FU-based chemotherapy.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Ref.</th>
<th>No. of pts</th>
<th>Stage of disease</th>
<th>Lesion tested</th>
<th>Method</th>
<th>% of MSI-H/ MMR protein neg</th>
<th>Adjuvant treatment</th>
<th>Better response to adjuvant 5-FU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemminki et al</td>
<td>2000</td>
<td>301</td>
<td>95</td>
<td>Duke's' C</td>
<td>CRC</td>
<td>PCR</td>
<td>12%</td>
<td>5-FU-based</td>
<td>MSI</td>
</tr>
<tr>
<td>Elsaleh et al</td>
<td>2000</td>
<td>300</td>
<td>656</td>
<td>Duke's' C</td>
<td>CRC</td>
<td>PCR</td>
<td>8.5%</td>
<td>5-FU-based</td>
<td>MSI</td>
</tr>
<tr>
<td>Ribic et al</td>
<td>2003</td>
<td>297</td>
<td>570</td>
<td>Stage II-III</td>
<td>Colon</td>
<td>PCR</td>
<td>16.7%</td>
<td>5-FU-based</td>
<td>MSS+MSI-L</td>
</tr>
<tr>
<td>Carethers et al</td>
<td>2004</td>
<td>298</td>
<td>204</td>
<td>Stage II-III</td>
<td>CRC</td>
<td>PCR</td>
<td>17.6%</td>
<td>5-FU-based</td>
<td>MSS+MSI-L</td>
</tr>
<tr>
<td>Benatti et al</td>
<td>2005</td>
<td>96</td>
<td>1263</td>
<td>Stage I-IV</td>
<td>CRC</td>
<td>PCR+IHC*</td>
<td>20.3%</td>
<td>5-FU-based</td>
<td>MSS+MSI-L</td>
</tr>
<tr>
<td>Storojeva et al</td>
<td>2005</td>
<td>304</td>
<td>160</td>
<td>NE</td>
<td>CRC</td>
<td>PCR</td>
<td>13.1%</td>
<td>5-FU-based</td>
<td>MSI-H=MSS+MSI-L</td>
</tr>
<tr>
<td>Jover et al</td>
<td>2006, 2009</td>
<td>302, 303</td>
<td>754</td>
<td>Stage II-III</td>
<td>CRC</td>
<td>PCR+IHC</td>
<td>10.1%</td>
<td>5-FU-based</td>
<td>MMR-competent</td>
</tr>
<tr>
<td>Kim et al</td>
<td>2007</td>
<td>305</td>
<td>542</td>
<td>Dukes'B+C</td>
<td>Colon</td>
<td>PCR</td>
<td>18.1%</td>
<td>5-FU-based</td>
<td>MSI-H=MSS+MSI-L</td>
</tr>
<tr>
<td>Sargent et al</td>
<td>2008</td>
<td>299</td>
<td>341</td>
<td>Stage II-III</td>
<td>Colon</td>
<td>PCR+IHC*</td>
<td>13.8%</td>
<td>5-FU-based</td>
<td>proficient MMR</td>
</tr>
<tr>
<td>Study IV</td>
<td>2010</td>
<td>1006</td>
<td>1006</td>
<td>Stage II-III</td>
<td>CRC</td>
<td>IHC</td>
<td>15.6%</td>
<td>5-FU-based</td>
<td>MMRPneg=MMRPpos</td>
</tr>
</tbody>
</table>

MMR; Mismatch repair, 5-FU; 5-Fluorouracil, pts; patients, NE; not evaluated, CRC; Colorectal cancer, PCR; polymerase chain reaction, IHC; immunohistochemistry, IHC*; Only performed in a subset of patients, MSI; Microsatellite instability, MSS; Microsatellite stability, MSI-L; Microsatellite instability low, MSI-H; Microsatellite instability high, MMRP; Mismatch repair protein.

The evaluation of MMR status as a predictive factor is particularly difficult in the metastatic setting as metastatic progression is a rare event for CRC tumours with MMR deficiency. In a systematic review and meta-analysis no benefit of chemotherapy was found in metastatic CRC in terms of response rate for MSI-H patients compared with MSS patients [308]. However, the heterogeneity among the included studies has to be considered with respect to difference in chemotherapy regimens as well as numbers of patients in each study. The MMR status was assessed retrospectively in all included studies also leading to various sources of bias.

The effect of 5-FU chemotherapy according to the MMR status in the primary tumour was investigated on stage IV patients, who underwent palliative surgery where patients with MSI-H, who received 5-FU-based chemotherapy had a significantly longer survival [309]. Brueckl et al. also found MSI-H assessed in the primary tumour to predict response to palliative 5-FU-based chemotherapy in first-line treatment of metastatic CRC [310]. These results contradict the results from the adjuvant setting on stage II-III CRC tumours, where 5-FU decrease the survival rate among patients with MSI-H tumours [96, 297-299, 303]. One hypothesis that might explain the divergent results according to treatment effects and MMR status could be an increased 5-FU sensitivity in metastatic cells, due to re-expression of MLH1/PMS2 [266]. A retrospective study found MSI-H to be a predictor for response to single treatment with irinotecan in patients with stage IV CRC, who previously failed to respond to 5-FU-based chemotherapy [311]. In stage IV CRC, MSI-H has...
also been reported to have a predictive value in patients treated with 5-FU in combination with irinotecan [312]. The treatment options in the metastatic setting are far more complex than the adjuvant setting. With the low number of MMR deficient tumours in metastatic CRC patients, MMR status as a single predictive marker seems to be of limited value. On the other hand, MMR-status might add valuable information in combination with other potential predictive markers. A summary of the studies from 2000-2008 evaluating MMR status as a predictive marker in metastatic CRC is listed in Table 6B [267,271,309-313].

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Ref.</th>
<th>No. of pts</th>
<th>Primary tumour</th>
<th>Metastatic site</th>
<th>Lesion tested</th>
<th>Method</th>
<th>% MSI-H/ MMRP neg</th>
<th>Treatment</th>
<th>Better response to treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosty et al</td>
<td>2001</td>
<td>271</td>
<td>56</td>
<td>CRC</td>
<td>Liver</td>
<td>Metastasis</td>
<td>PCR</td>
<td>1.8%</td>
<td>5-FU-based</td>
<td>MSSI-H=MSS+MSI-L</td>
</tr>
<tr>
<td>Liang et al</td>
<td>2002</td>
<td>309</td>
<td>244</td>
<td>CRC</td>
<td>Lung, Liver, Other</td>
<td>Primary</td>
<td>PCR</td>
<td>21.3%</td>
<td>5-FU-based</td>
<td>MSSI-H</td>
</tr>
<tr>
<td>Fallik et al</td>
<td>2003</td>
<td>311</td>
<td>72</td>
<td>CRC</td>
<td>Local, Liver</td>
<td>Primary/ Metastasis</td>
<td>PCR+IHC*</td>
<td>10.8%</td>
<td>Irinotecan</td>
<td>MSSI-H</td>
</tr>
<tr>
<td>Brueckl et al</td>
<td>2003</td>
<td>310</td>
<td>43</td>
<td>CRC</td>
<td>Local, Lung, Liver</td>
<td>Primary</td>
<td>PCR+IHC*</td>
<td>16%</td>
<td>5-FU-based</td>
<td>MSSI-H</td>
</tr>
<tr>
<td>Bendardaf et al</td>
<td>2007</td>
<td>312</td>
<td>73</td>
<td>CRC</td>
<td>Local, Lung, Liver</td>
<td>Primary</td>
<td>IHC</td>
<td>NE</td>
<td>5-FU+ Irinotecan</td>
<td>MMRP negative</td>
</tr>
<tr>
<td>Des Guetz et al</td>
<td>2007</td>
<td>313</td>
<td>40</td>
<td>CRC</td>
<td>LN, Local, Lung, Liver</td>
<td>Primary</td>
<td>PCR</td>
<td>22%</td>
<td>FOLFOX4/ FOLFOX6</td>
<td>MSSI-H=MSS</td>
</tr>
<tr>
<td>Müller et al</td>
<td>2008</td>
<td>267</td>
<td>108</td>
<td>CRC</td>
<td>NE</td>
<td>Primary/ Metastasis</td>
<td>PCR+IHC</td>
<td>4%</td>
<td>FUFOX/ CAPOX</td>
<td>MSSI-H=MSS+MSI-L</td>
</tr>
</tbody>
</table>

MMR: Mismatch repair, pts: patients, CRC: Colorectal cancer, LN: lymph node, NE: not evaluated, PCR: polymerase chain reaction, IHC: immunohistochemistry, IHC*: Only performed in a subset of patients, 5-FU: 5-Fluouracil, FOLFOX: 5-Fluouracil+Oxaliplatin, MSI-H: Microsatellite instability high.

### 5.3 EGFR (Epidermal Growth Factor Receptor)

EGFR belongs to the erbB family of receptor tyrosin kinases and is an important target for cancer treatment as its activation stimulates processes involved in tumour growth and progression such as proliferation, angiogenesis, invasion and metastasis. EGFR expression in CRC has been reported in the literature to range from 16% for early cancer to 80% in advanced disease [314,315]. The binding of a ligand to EGFR initiates different downstream signaling pathways for example the RAS-RAF-mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase and phospholipase C (PI3KCA)-AKT pathway, (Figure 8) [316]. Cetuximab and panitumumab are monoclonal antibodies binding to EGFR with greater affinity than the natural ligand EGF. This competitive inhibition is preventing receptor dimerization, phosphorylation and downstream signaling that finally leads to apoptosis of the cancer cells. Initially positive EGFR expression determined by IHC
was a criterion for enrolment in clinical trials evaluating the effect of EGFR inhibitors. However, objective responses were found in patients independently of EGFR status that led to intense research in order to define other potential markers that correlate with an improved response to cetuximab and panitumumab. Research demonstrated that mutation in KRAS, BRAF, or PI3KCA results in continuous activation of the downstream RAS-MAPK or PI3KCA-AKT pathways, regardless of whether the ligand EGF binds to EGFR or the receptor is pharmacologically blocked. Mutations in KRAS are most common and occur in 35-45% of the CRC tumours, whereas mutations in BRAF (< 15%) and PI3KCA (≤ 20%) are less common [317-319]. Mutations in PI3KCA and KRAS or BRAF can be found together in the same tumour but mutations in KRAS and BRAF do not seem to coexist [39,320], (Figure 9).

Figure 8. Overview of interlinked cellular signaling pathways involved in the proliferation and progression of colorectal cancer [321]. The epidermal growth factor receptor (EGFR)–related family of receptor tyrosine kinases includes human epidermal growth factor receptor (HER1), EGFR, or c-erbB1; HER2 or c-erbB2; HER3 or c-erbB3; and HER4 or c-erbB4. C-MET = mesenchymal–epithelial transition factor; EGF = epidermal growth factor; HDAC = histone deacetylases; HGF = hepatocyte growth factor; IGF-1 = insulin-like growth factor-I; IGF-1R = insulin-like growth factor-I receptor; IR = insulin receptor; VEGF = vascular endothelial growth factor; VEGF-R = vascular endothelial growth factor receptor. Agents targeting signaling proteins that have been evaluated or are currently being evaluated in phase II, III, or IV clinical trials for colorectal cancer are shown. Reproduced with permission from J Natl Cancer Inst. 2009 Oct 7;101(19):1308-24. ©The Author 2009. Published by Oxford University Press.
**KRAS**

The somatic KRAS missense mutations found in cancer introduce substitutions of amino acids at positions 12 and 13 but less commonly at position 61 and 146, resulting in an accumulation of the KRAS protein in an active GTP-bound conformation. The prognostic value of KRAS mutations has been investigated independently of anti-EGFR treatment. In phase III monotherapy studies with either cetuximab or panitumumab KRAS mutations did not have any prognostic value among patients receiving only best supportive care [322-324]. In the Pan-European Trials in Adjuvant Colon Cancer (PETACC) 3 trial, no association was found between KRAS mutations and relapse-free survival or OS in stage II and III colon cancer indicating no stage-specific prognostic value [325].

The first large study that confirmed the negative predictive value of KRAS mutations was the pivotal randomized phase III study comparing panitumumab monotherapy with best supportive care [324]. Thereafter, several trials have confirmed this finding [167,168,323]. Based on a systematic review of the literature the ASCO provisional clinical opinion has declared that all patients with metastatic CRC, who are eligible for treatment with an anti-EGFR monoclonal antibody should have their tumours tested for KRAS mutations before therapy is started [326]. If a KRAS mutation is found in either codon 12 or 13 the patients should not receive anti-EGFR monoclonal antibody therapy. The identification of wild-type KRAS as a predictive marker for cetuximab and panitumumab is an important step towards a more individualized treatment approach in metastatic CRC.

**BRAF**

Among patients with KRAS wildtype metastatic CRC about 60% do not respond to cetuximab or panitumumab. BRAF is located downstream from KRAS in the MAPK pathway and mutations in the BRAF gene occur in approximately 5-10% of the patients with KRAS wildtype tumours. Data suggests that the BRAF mutation, mainly the somatic Val600Glu (V600) mutation, is responsible for part of the EGFR antibody resistance [327].

In a retrospective study with metastatic CRC patients (n=113) treated with either cetuximab or panitumumab the BRAF V600 mutation was detected in 11 of 79 (13.9%) patients with wild-type KRAS tumours [328]. None of the responders carried BRAF mutations. BRAF-mutated patients also had significantly shorter PFS and OS than wild-type patients. Furthermore, treatment with the multikinase inhibitor sorafenib restored sensitivity to cetuximab and panitumumab in BRAF-mutated CRC cell lines.

BRAF mutations have also been reported to occur more frequently in sporadic colon cancer patients, whose tumours are MMR deficient due to promoter hypermethylation of MLH1 [39]. Another study has shown that a combined analysis
of MMR status and BRAF mutation status in colon cancer improves the prognostic information compared to separate analysis of each factor [329].

**PI3KCA-AKT and PTEN**

Resistance to EGFR inhibitors could also be a result of mutations in the PI3KCA-AKT pathway directly or as a loss of expression of the tumour suppressor PTEN protein [327]. Additional studies are needed in order to evaluate the role of PIK3CA mutations and loss of PTEN as predictive markers for response to EGFR-inhibitors.

**Figure 9.** Potential relationship between KRAS status and response to epidermal growth factor receptor (EGFR) monoclonal antibodies [327]. Reprinted with permission J Clin Oncol. 2008 Dec 10;26(35):5668-70. Copyright 2008 © American Society of Clinical Oncology.

### 5.4 Loss of heterozygosity at 18q (18q LOH)

An allelic loss on chromosome 18q occurs in approximately 70% of all CRCs, but is less common in patients with MSI CRC [10,23]. The gene DCC (deleted in CRC) is located at 18q and codes for a neutr-in-1 receptor, that is implicated in colorectal tumourigenesis with involvement in apoptosis, cell adhesion, and tumour suppression [17]. Other genes located in this chromosomal region include SMAD2 and SMAD4/DPC [18,19]. Both SMAD2 and SMAD4 are involved in the TGF-β signaling pathway and mutations in these genes have been found in CRC.

There are several techniques used to assess LOH at 18q [330]. The most commonly used assay examines polymorphisms at multiple microsatellites on 18q with genotyping. Another frequently used technique is IHC where DCC protein status is defined with a monoclonal or polyclonal antibody. A systematic review and meta-analysis suggested that CRCs with LOH at 18q have an impaired prognosis but emphasized the need for prospective studies using consistent methodology to better evaluate its prognostic role [330]. Furthermore, in a recently published report with non-MSI-high colorectal cancers (stage I to IV) from two independent prospective cohort studies 18q LOH was not associated with survival [331].
Watanabe et al. have reported that retention of 18q alleles in MSS cancers and mutation of the gene for the type II receptor for TGF-β1 in MSI-H cancers is associated with a favourable outcome after adjuvant chemotherapy with 5-FU-based chemotherapy for stage III colon cancer [332]. The benefit of these markers in stage II colon cancer patients has not been shown. This question is being addressed in an ongoing prospective Intergroup E5202 study, where stage II colon cancer patients are stratified based on the presence of MSI and LOH of the 18q allele. Low-risk patients are observed, whereas high-risk patients (MSS with 18qLOH and MSI-L with 18qLOH) are randomized to FOLFOX with or without bevacizumab [333]. Results from this study will hopefully contribute to a better understanding of the role of LOH at 18q and MMR status as predictive factors in colon cancer.

However, other studies have reported contradictory results regarding the response to adjuvant 5-FU with either an improved survival in 18q LOH patients or no survival difference at all between patients with LOH at 18q and patients with an intact 18q [182]. Further prospective validation of LOH at 18q as a predictive marker in CRC is therefore needed.

5.5 **TP53**

TP53 is a tumour suppressor gene with a central role in many apoptotic pathways. Mutations in TP53 exist in up to 70% of all sporadic CRCs and seem to occur late in the genesis of CRC [20,21]. TP53 mutations occur in MSI CRC tumours but in a lower frequency than in CIN tumours [334]. The prognostic and predictive value of TP53 has been investigated with conflicting results. At present there is no standardized assay available to determine TP53 status in CRC and various methods are used for example IHC to detect TP53 protein expression or DNA sequence analysis of gene mutations. A systematic review of 168 studies with data in a total of 18,766 patients showed that patients with TP53 abnormalities were at an increased risk of death but an abnormal TP53 did not predict outcome in patients treated with chemotherapy [335]. Furthermore, an analysis of a cohort of 3583 CRC patients found that patients with wildtype TP53 receiving adjuvant treatment had a significant better survival than patients treated with surgery alone, regardless of tumour site [336].

5.6 **Dihydropyrimidine Dehydrogenase (DPD) and Thymidine Phosphorylase (TP)**

DPD is an enzyme that occurs mainly in the liver and is responsible for the catabolism of 5-FU [337]. A profound systemic toxicity can occur in 5-FU treated patients with DPD deficiency [338]. In CRC, DPD alone does not seem to be a strong prognostic marker and there is only limited data supporting its role as a predictor of
response to chemotherapy [182,339]. However, inhibition of DPD has been proposed as a strategy to increase the efficacy of 5-FU as in the treatment with UFT, that consist of the DPD inhibitor uracil and the 5-FU prodrug tegafur [214].

TP is involved in the conversion of 5-FU to the active metabolite FdUMP that inhibits TS and is also responsible for the last step in the enzymatic activation of the oral 5-FU prodrug, capecitabine. Furthermore, TP is also known as the platelet-derived endothelial cell growth factor (PD-ECGF), which might have an angiogenic function [340]. In comparison with normal tissue the expression of TP is higher in tumour cells and in tumour infiltrating stromal cells [212,341]. The complex biological functions of TP make it difficult to evaluate its potential prognostic and predictive role in CRC [182,339].
6 AIMS OF THE THESIS

To analyze:

- whether TS expression in lymph node metastases correlates with the expression of TS in their corresponding primary tumours and if TS expression in lymph node metastases has a prognostic accuracy and can predict outcome of adjuvant 5-FU-based chemotherapy in stage III colorectal cancer.

- the expression of TS in liver metastases and lung metastases of colorectal cancer and their matched primary tumours.

- the value of TS expression in the primary tumour of stage II and III colorectal cancer as a prognostic and predictive marker in patients treated with adjuvant therapy according to the dose of 5-FU.

- the role of MMR protein expression in stage II and III colorectal cancer as a prognostic marker and a predictive marker in patients treated with 5-FU based adjuvant chemotherapy.

- if a combined analysis of MMR protein expression and TS expression in stage II and III colon cancer can improve the prediction of clinical outcome and response to adjuvant 5-FU-based chemotherapy and whether there is a correlation between MMR protein expression and TS expression.
7 PATIENTS AND METHODS

7.1 Patients

Patient characteristics for study I-V are listed in Table 7. In our studies we used the Dukes’ pathological classification of stage in study I-II and the 6th edition of the AJCC staging system in study III-V.

Table 7. Patient and tumour characteristics Study I-V.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Study I</th>
<th>Study II</th>
<th>Study III</th>
<th>Study IV</th>
<th>Study V</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of tumours aimed to analyze</td>
<td>423</td>
<td>45*/38**/10***</td>
<td>1389</td>
<td>1042</td>
<td>718</td>
</tr>
<tr>
<td>No. of analyzed tumours</td>
<td>348</td>
<td>45*/38**/10***</td>
<td>1389</td>
<td>1006</td>
<td>716</td>
</tr>
<tr>
<td>Median age (range)</td>
<td>64 (31-75)</td>
<td>63 (38-80)</td>
<td>65 (29-75)</td>
<td>66 (24-75)</td>
<td>66 (24-75)</td>
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<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>202</td>
<td>26</td>
<td>782</td>
<td>559</td>
<td>379</td>
</tr>
<tr>
<td>Female</td>
<td>146</td>
<td>19</td>
<td>607</td>
<td>447</td>
<td>337</td>
</tr>
<tr>
<td>Tumour site</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>232</td>
<td>24</td>
<td>984</td>
<td>718</td>
<td>716</td>
</tr>
<tr>
<td>Rectum</td>
<td>116</td>
<td>21</td>
<td>405</td>
<td>288</td>
<td>~</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>Dukes’ C</td>
<td></td>
<td></td>
<td>Stage II (678)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dukes’ D</td>
<td></td>
<td></td>
<td>Stage III (711)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td></td>
<td></td>
<td>Stage II (488)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage III (356)</td>
<td></td>
<td></td>
<td>Stage III (518)</td>
<td></td>
<td></td>
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<tr>
<td>Stage III (360)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastatic site</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph nodes</td>
<td></td>
<td>Lung, Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph nodes</td>
<td></td>
<td></td>
<td>Lymph nodes</td>
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<td>Lymph nodes</td>
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<td>Lymph nodes</td>
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<td>Lymph nodes</td>
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<tr>
<td>Adjuvant CT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With</td>
<td>170</td>
<td>5</td>
<td>681</td>
<td>491</td>
<td>346</td>
</tr>
<tr>
<td>Without</td>
<td>178</td>
<td>40</td>
<td>708</td>
<td>515</td>
<td>370</td>
</tr>
<tr>
<td>TS-grade (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (0-1)</td>
<td>47%</td>
<td>Primary (19%)</td>
<td>29%</td>
<td>~</td>
<td>~</td>
</tr>
<tr>
<td>High (2-3)</td>
<td>57%</td>
<td>Primary (81%)</td>
<td>71%</td>
<td>~</td>
<td>~</td>
</tr>
<tr>
<td>TS-grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (0-2)</td>
<td>~</td>
<td>~</td>
<td>67%</td>
<td>~</td>
<td>62%</td>
</tr>
<tr>
<td>High (3)</td>
<td>~</td>
<td>~</td>
<td>33%</td>
<td>~</td>
<td>38%</td>
</tr>
<tr>
<td>MMR-status (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMRP neg</td>
<td>~</td>
<td>~</td>
<td>~</td>
<td>15.6%</td>
<td>20%</td>
</tr>
<tr>
<td>MMRP pos</td>
<td>~</td>
<td>~</td>
<td>~</td>
<td>84.4%</td>
<td>80%</td>
</tr>
</tbody>
</table>

No.; Number, Adjuvant CT; Adjuvant chemotherapy, TS; Thymidylate synthase, MMR; Mismatch repair, MMRP; Mismatch repair protein, *; Primary tumour, **; Liver metastases, ***; Lung metastases.
Patients and methods

Study I and III-V

The Nordic colorectal group enrolled 2224 patients with CRC (Dukes’B and Dukes’C) in adjuvant clinical trials from 1991 to 1996 [342]. Only patients up to the age of 75 years and with no history of other malignancies within the last five years were included. The patients were randomised between surgery alone or surgery plus adjuvant chemotherapy with a regimen containing 5-FU according to the Moertel schedule with levamisole [343], the Mayo regimen with or without levamisole [344] or the Nordic schedule with or without levamisole [345]. The adjuvant chemotherapy was started within 11 weeks after surgery.

In a previous study from our group with 862 patients from Sweden and Denmark of the total 2224 CRC patients enrolled in the Nordic adjuvant trials, TS expression was determined in primary tumours using IHC [227]. Among the 862 previous trial participants 423 patients (49%) had Dukes’ C CRC. In study 1, the expression of TS was defined with IHC on tissue samples from primary tumours and lymph node metastases that were available from 348 (82 %) of the 423 patients with Dukes’ C CRC. These tumour samples were obtained from 28 hospitals in Sweden and 2 hospitals in Denmark. In study III, IHC analysis of TS expression on tumours from another 527 Swedish CRC patients were added to the 862 Swedish and Danish CRC patients where TS already had been analyzed, which resulted in a total study cohort of 1389 patients obtained from a total of 52 hospitals. In study IV, we determined MMR protein expression (MLH1 and MSH2) with IHC on 1042 patients of the 1389 CRC patients reported in study III. Thirty-six (3.4%) of the 1042 patients were excluded from the study because the quality of the immunostaining was unsatisfactory and this study reports the result from analyses of the remaining 1006 patients. In study V, we evaluated a combined analysis of TS expression and MMR protein expression (MLH1 and MSH2) in a subgroup of 716 Swedish colon cancer patients, who also were included in study III and IV.

The number of obtainable tumour specimens available for analyses of TS expression and MMR protein expression is solely a result of the tumour material that was submitted by the treating institutions. Parameters of clinical outcome were obtained from 6 regional centres of epidemiological oncology in Sweden for patients in Study I and III-V. For study I and III outcome data of the included Danish patients was collected from one centre of epidemiological oncology in Denmark.

Study II

The study population consists of 45 patients with primary CRC and corresponding liver metastases (n=38) and lung metastases (n=10). Three patients had both liver metastases and lung metastases. The patients underwent surgery for primary CRC and radical resection for metastatic disease from 1987 to 1997 at the Southern Hospital in
Patients and methods

Stockholm, Sweden. Outcome data was collected from the registry at the National Board of Health and Welfare.

7.2 Laboratory methods

IHC techniques have been used to semi-quantitatively determine TS protein expression and MMR protein expression of MLH1 and MSH2 respectively. We also performed a few analyses of the MMR protein, PMS2, in order to verify MLH1 protein deficiency because of the functional interaction between PMS2 and MLH1, (Figure 7). The monoclonal antibody, TS106, was used to detect TS expression [223]. There is also a polyclonal rabbit-antihuman TS antibody available, but data has not revealed any major differences in the detection of TS expression between these two antibodies [224]. The antibodies and dilutions used are listed in Table 8.

Table 8. Summary of the antibodies used for immunostaining.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Clone</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS Ab-1</td>
<td>NeoMarkers, Fremont, CA, USA</td>
<td>TS106</td>
<td>1:75</td>
</tr>
<tr>
<td>MLH1</td>
<td>PharMingen, San Diego, CA, USA</td>
<td>G 168-15</td>
<td>1:100</td>
</tr>
<tr>
<td>MSH2</td>
<td>Oncogene, Research Products, Boston, MA, USA</td>
<td>FE-11</td>
<td>1:100</td>
</tr>
<tr>
<td>PMS2</td>
<td>PharMingen, San Diego, CA, USA</td>
<td>A 16-4</td>
<td>1:75</td>
</tr>
</tbody>
</table>

Figure 10. Schematic illustration of the avidin-biotin-peroxidase complex (ABC) technique. A primary antibody (TS106) is incubated with the antigen of interest (TS). When the secondary biotinylated antibody binds to the primary antibody, peroxidise reacts with biotin which leads to visible staining.
Patients and methods

TS protein immunostaining and scoring

The method used is a slight modification of the TS protein immunostaining method described by Johnston et al. [223]. The specimens derived from formalin-fixed paraffin-embedded tumours in which two sections, each 4 μm thick, were taken from different parts of the tumour. The analysis of TS protein expression was carried out using the standard avidin-biotin-peroxidase complex (Vectasin Elite ABC kit; Vector Laboratories, Inc; Burlingame, CA, USA) [346], Figure 10. The tumour sections were deparaffinized in xylene and then rehydrated in graded ethanol. Endogenous peroxidase activity was quenched with 3 % hydrogen peroxide in distilled water for 10 minutes.

Antigen retrieval was done in citrate buffer (pH 6.0) by cooking for 20 minutes in a microwave oven followed by 20 minutes of cooling at room temperature. In order to reduce non-specific background staining the slides were blocked with horse serum followed by an overnight incubation at 4°C+ with the TS106 monoclonal antibody. The samples were then rinsed and incubated with biotinylated horse anti-mouse secondary antibodies and thereafter rinsed and incubated with avidin-biotin-peroxidase complexes. Visualization of immunostaining was achieved by immersion in 0.05% 3,3’-diaminobenzidine tetrahydrochloride followed by counterstaining with hematoxylin.

The monoclonal antibody TS106 produces a granular cytoplasmatic staining pattern. There is a minor cross-reactivity with smooth muscle cells. TS staining can also be found in lymphocytes and macrophages adjacent to the tumour. The normal colon epithelium shows a weak or no staining. TS staining intensity is defined by a visual grading scale from 0-3 [225]. Low intensity staining of TS has historically been defined as 0 and 1 and high intensity staining of TS as 2 and 3 [226], (Figure 11A-D). In study III, we added another categorization, where the intensity of TS expression is classified as low for TS grade 0-2 and as high for TS grade 3, a categorization that was also used in study V [228].

Two reference slides were included each time a set of tumour samples was stained. The TS grade was based on the highest intensity found in the tumour, even if the high-staining area was small. Our group has previously reported that the probability of finding an area of maximum TS staining with a single tumour sample is 81% and with two tumour samples the percentage rise to 96% [200]. Due to the heterogeneity with respect to TS expression, tissue samples from two different areas of each tumour were analyzed. Three or four of the authors have performed the scoring in each study blinded to clinical data, in study I-II (DE, MH, KÔ) and in study III plus V (KÔ, MK, DE, MH). The level of agreement between the observers was ≥ 90%. Scoring discrepancies were resolved by consensus after re-examination.
Patients and methods

A. TS grade 0  B. TS grade 1

C. TS grade 2  D. TS grade 3

Figure 11 A-D. Immunohistochemical detection of the TS protein on paraffin sections of primary colorectal cancer.

**MMR protein immunostaining and scoring**

Intratumoural heterogeneity in MMR protein expression has been described and therefore two formalin-fixed paraffin-embedded, tissue samples from different parts of each resected primary tumour were used for study IV and V [347]. 4 µm sections were mounted on SuperFrost Plus glass slides dried at room temperature and then baked for 1 hour in 60°C, deparaffinized in xylene, rehydrated in graded ethanol, and washed in distilled water. Thereafter the slides were incubated in a 3% hydrogen peroxide to inhibit the endogenous peroxidise activity. Antigen retrieval was achieved by microwave-treatment for 20 minutes in 1mM EDTA (pH 9.0) and afterwards cooled down for 20 min in the same buffer at room temperature. The slides were incubated with a mouse immunoglobulin G monoclonal primary antibody for either MLH1, MSH2 or PMS2 overnight in 4°C, (Table 8) and thereafter incubated with an amplification system with a labelled polymer, EnVision™/HRP rabbit/mouse and goat/rabbit, (DakoCytomation, Denmark) for 30 minutes. The EnVision™ system is a two step staining technique in which the primary antibody is followed by a polymeric conjugate in sequential steps that can eliminate the problem of endogenous biotin [348]. The polymeric conjugate consists of many peroxidase molecules and secondary antibody molecules bound directly to an activated dextran backbone. To visualize the sites of bound peroxidase 0.05 % 3, 3’-diaminobenzidine tetrahydrochloride (DAB) was used before counterstaining with a modified Harris hematoxylin.

All tumour samples were examined under a light microscope and scored by two independent observers blinded to clinical and pathological data, in study IV and V (KÖ, MH, DE). The agreement in evaluation between the observers was > 90 %. In case of disagreement the final score was determined by consensus after re-examination. A case was considered negative for expression of a given protein if the tumour cells displayed a complete absence of nuclear staining with the respective monoclonal antibody, (Figure 12A-B). Intact nuclear staining of normal tissue (non-neoplastic stromal cells and lymphocytes) adjacent to the tumour was used as internal positive control. If nuclear staining was not obtained in the internal control tissue the sections were classified as non-evaluable. We scored a case as positive for
immunostaining if any area of the tumour exhibited a positive staining. The MSH2 antibody staining showed a nuclear staining that was easy to interpret. However, the MLH1 antibody yielded a few cases with a patchy staining, which has also been described by others [286,349]. There is a functional interaction between the MMR proteins MLH1 and PMS2 as well as between MSH2 and MSH6, (Figure 7). It has been suggested that the PMS2 and MSH6 proteins are degraded if they do not have their binding partners. For example, in most tumours with a MLH1 mutation there is also a loss of PMS2 protein expression [350]. In the screening of HNPCC it is therefore recommended to include PMS2 and MSH6 antibodies in the routine test panel. Whether staining with the PMS2 antibody can give additional information in sporadic CRC if the MLH1 staining is hard to evaluate, has to be further investigated as the loss of protein expression of MLH1 in these cases is due to promoter methylation. In study IV, the MLH1 staining was hard to interpret in 51 cases, where we also added determination of PMS2 and if negative the MLH1 staining was classified as negative.

![A. MLH1 negative](image1.png)  ![B. MLH1 positive](image2.png)

**Figure 12 A-B.** Immunohistochemical detection of the MLH1 protein on paraffin sections of primary colorectal cancer.

### 7.3 Statistical methods

Statistical analyses were performed using STATISTICA release 7, (StatSoft®, Scandinavia AB, Uppsala, Sweden). All statistical tests were two-sided and used a p-value of < 0.05 for statistical significance.

The Chi-square test of association was used to calculate the differences in distribution between groups (study I-V). The Spearman’s rank test (study II and V) and the Pearson correlation coefficient (study II), were performed to analyze the correlation of variables between groups. To define the difference with respect to TS in distant metastases and their matched primary tumours we used the Sign test of discordant pairs in a cross-table (study II). The Gehan Wilcoxon univariate test was used to examine the possible correlation between OS and DFS with respect to patient as well as tumour characteristics and TS expression and MMR expression respectively (study I, III-V) [351]. OS was defined as time from surgery to death and DFS was defined as time from surgery to the first event of local recurrence, presence of distant metastases or death of any reason. In study I, we also analyzed cancer-specific survival (CSS) defined as the interval from the date of surgery to the date of death in CRC. Survival distributions were estimated using the Kaplan-Meier method (study I and III-V) [352]. Multivariate analyses were performed using Cox regression models (study I, III-V), [353].
8 RESULTS AND DISCUSSION

8.1 Study I

The aims of this study were to define whether TS expression in lymph node metastases correlates with TS expression in the primary tumour and to evaluate if TS expression in lymph node metastases has a prognostic value and can predict outcome of adjuvant 5-FU chemotherapy.

In the adjuvant setting assessment of prognostic and predictive markers is mainly based on analysis of the primary tumour. However, the primary tumour is often heterogeneous and the characteristics of the primary tumour can be different compared to the characteristics found in lymph node metastases.

Study I included 348 patients with Dukes’C CRC and is to our knowledge the largest randomized study assessing TS in lymph node metastases of CRC and corresponding primary tumours yet published [206,252,354-356], (Table 9). The patients had all been enrolled onto a previous study of 862 CRC patients, who were included in Nordic trials randomly assigned to surgery alone or surgery plus 5-FU-based chemotherapy [227]. TS expression was analysed using IHC in the primary tumour as well as in the lymph node metastases for all patients. The patients in this study underwent surgery between 1991-1996, when the impact of examining a high number of lymph nodes was still not clear. The median number of lymph nodes found in the surgical specimens was five (range, 1-20) and the median number of lymph nodes containing tumour cells was two (range, 1-20). The limited number of lymph nodes might have had an impact on the categorization of the TS cohorts.

Table 9. The expression of TS in lymph node metastases and matched primary tumours.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Ref</th>
<th>No. of pts</th>
<th>Primary tumour</th>
<th>Stage of disease</th>
<th>Method</th>
<th>TS Primary</th>
<th>TS LN</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edler et al</td>
<td>2000</td>
<td>354</td>
<td>243 (56*)</td>
<td>Rectal</td>
<td>Dukes’A-C</td>
<td>IHC (m)</td>
<td>NE</td>
<td>NE</td>
<td>Yes (p&lt; 0.001)</td>
</tr>
<tr>
<td>Yamada et al</td>
<td>2001</td>
<td>206</td>
<td>90 (12**)</td>
<td>CRC</td>
<td>NE</td>
<td>PCR</td>
<td>Median TS(GADPH) mRNA ratio: 0.98</td>
<td>Median TS(GADPH) mRNA ratio: 1.92</td>
<td>No (Higher in LN p&lt; 0.001)</td>
</tr>
<tr>
<td>Berglund et al</td>
<td>2002</td>
<td>252</td>
<td>119 (39*)</td>
<td>CRC</td>
<td>Dukes’A-D</td>
<td>IHC (m)</td>
<td>High TS: 78%</td>
<td>NE</td>
<td>Yes</td>
</tr>
<tr>
<td>Marsh et al</td>
<td>2002</td>
<td>355</td>
<td>42</td>
<td>CRC</td>
<td>Dukes’C-D</td>
<td>IHC (m)</td>
<td>High TS: 68%</td>
<td>High TS: 81%</td>
<td>No (Higher in LN)</td>
</tr>
<tr>
<td>Study I</td>
<td>2005</td>
<td>348</td>
<td>CRC</td>
<td>Dukes’A-C</td>
<td>IHC (m)</td>
<td>High TS: 74%</td>
<td>High TS: 67%</td>
<td>Yes (p&lt; 0.001)</td>
<td></td>
</tr>
<tr>
<td>Vallböhmer et al</td>
<td>2006</td>
<td>356</td>
<td>35</td>
<td>CRC</td>
<td>NE</td>
<td>PCR</td>
<td>T5x100/β-actin:1.8</td>
<td>T5x100/β-actin:1.7</td>
<td>Yes (p&lt; 0.001)</td>
</tr>
</tbody>
</table>

TS; Thymidylate synthase, No.; Number, pts; patients, *; Numbers of available corresponding metastases, CRC; Colorectal cancer, LN; Lymph nodes, NE; Not evaluated, IHC(m); Immunohistochemistry monoclonal antibody, PCR; Polymerase Chain Reaction.

A high expression of TS was found in 67% of the lymph node metastases and in 74% of the matched primary tumours. The number of available tumour samples for TS
analyses from each patient is a potential source of bias as the possibility of detecting areas of high TS expression would increase in proportion to the number of analyzed tumour samples. There was a significant correlation between the number of analyzed tumour samples and TS expression in the lymph node metastases (p=0.03) as well as TS expression in the primary tumours, (p=0.04). However, the mean number of lymph node metastases was the same in the group of patients with high TS expression as well as low TS expression in the primary tumour.

Even if a difference in TS expression was seen in the lymph node metastases and the matched primary tumour in 28% of the patients there was a significant correlation between the expression of TS in the lymph node metastases and TS in the primary tumour, (p=0.001). This result is in accordance with three other reports [252,354,356]. Two additional reports have failed to show a correlation and demonstrated instead a significantly higher TS expression in the lymph node metastases compared with the matched primary tumours [206,355]. When comparing the results from different studies it is important to be aware of the lack of standardization of the various methods (IHC and PCR) used to assess and evaluate TS expression.

We also found that in patients with a low TS expression in the primary tumour (n=92), TS expression in the lymph node metastases was an additive significant prognostic marker for cancer-specific survival (CSS), (p=0.02).

TS expression in the primary tumour as well as in distant metastases is a prognostic indicator, where patients with a high expression of TS in their tumours have a worse prognosis which is described in a meta-analysis [220].

TS expression in lymph node metastases as a prognostic marker in CRC is not well studied. In study I, we demonstrated that TS in the lymph node metastases was a prognostic factor in the entire study group with regard to OS (p=0.02) and DFS (p=0.04), where a low TS expression correlated with a longer OS and DFS. TS expression in lymph node metastases also had a significant prognostic value in multivariate analysis with respect to OS (p=0.02) and DFS (0.04), independently of age, gender, site of tumour and treatment. In subgroup analysis, TS in lymph node metastases was a significant prognostic marker in patients treated with surgery alone for OS (p=0.04) and DFS (p=0.03), but not in patients who received adjuvant chemotherapy. One possible explanation could be the potential beneficial effect of adjuvant 5-FU-based chemotherapy that reduces the survival differences between patients with high TS versus low TS in their lymph node metastases. Furthermore, we did not consider other clinicopathological risk factors that could have affected prognosis. There was no clinical benefit of adjuvant 5-FU based chemotherapy in this study group, which is in contrary with the general consensus that there is a significant survival advantage with adjuvant chemotherapy in stage III disease [358]. The primary aim of study I was not to evaluate the effect of 5-FU-based chemotherapy. This will instead be further discussed in study III and V.
In this study, TS expression in the primary tumour only had a significant prognostic value among patients who were treated with surgery alone (OS, \(p=0.03\); DFS, \(p=0.03\)). In the treatment of CRC a high TS expression in the primary tumor seems to be predictive for a better response to adjuvant 5-FU-based chemotherapy, (Table 5A), \([219,225,230,236,237,239, \) Study III]. However, the potential use of TS as a predictor of benefit from adjuvant 5-FU still remains unclear, as there are other studies suggesting that TS does not have any predictive value \([226-228,240,241]\).

In metastatic CRC, a majority of studies have described an association between a low TS expression in the distant metastases and a better response to 5-FU (Table 5B) \([203,229,233-235,244-250]\). Although the expression of TS in distant metastases of CRC may be a better predictor of response to 5-FU-based chemotherapy than TS-expression in the primary tumour, the predictive role of TS expression in lymph node metastases has to be further evaluated.

The results from studies including more than a hundred patients analyzing if chemosensitivity to 5-FU at metastatic sites can be predicted by TS expression in the primary tumour, are contradictory and need additional evaluation \([252,253,255,258]\).

In study I, we found a significant correlation between the expression of TS in the lymph node metastases and their matched primary tumour. We could also demonstrate that TS expression assessed using IHC in lymph node metastases of CRC improves the prognostic precision compared with TS expression defined in the primary tumour, but does not predict response to 5-FU-based adjuvant chemotherapy.

The potential role of TS expression in lymph node metastases as prognostic marker in stage III CRC has to be confirmed in a treatment setting with a higher number of lymph nodes examined (more than 12) according to the present guidelines \([116,117]\).

### 8.2 Study II

The main aim of this study was to analyze TS expression in liver metastases (n=38) and lung metastases (n=10) and the expression of TS in their corresponding primary tumours (n=45) in CRC patients who underwent radical metastatic surgery. Three patients had both liver metastases and lung metastases.

A high TS expression was seen in 39/48 distant metastases (81%) and in 36/45 (80%) of the primary tumours. Among the 39 patients with a high TS expression in their distant metastases 31/39 (79%) also had a high TS expression in their primary tumours, (Table 10). However, there was no significant correlation between TS expression in distant metastases and their matched primary tumours \((r=0.02, \text{ Spearman's rank test})\). In eight of fifteen discordant cases, the expression of TS was non-significantly higher in the distant metastases compared to the primary tumours.
Table 10. TS in distant metastases (n=48) as compared to TS in their matched primary tumours (n=45).

<table>
<thead>
<tr>
<th>TS-expression</th>
<th>No. of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distant metastases</td>
<td>Matched primary</td>
</tr>
<tr>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>H</td>
<td>L</td>
</tr>
<tr>
<td>L</td>
<td>H</td>
</tr>
<tr>
<td>L</td>
<td>L</td>
</tr>
</tbody>
</table>

H, High TS expression; L, Low TS expression.

To date 14 reports have compared TS expression in distant metastases and their matched primary tumours. Eight reports have used IHC (Table 11A) [201-204,244,245,359, Study II] and six reports PCR (Table 11B) [205,206,360-363] to assess TS expression.

Among the eight reports where IHC was used to assess TS expression four reports found a significant different TS in distant metastases compared with the matched primary tumours, [201-204]. Two reports found higher TS in the metastases while two reports described lower TS in the metastases.

Table 11A. TS expression (IHC) in distant metastases compared to matched primary tumours.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Ref</th>
<th>No. of pts</th>
<th>Metastatic site</th>
<th>% High TS Metastasis</th>
<th>% High TS Primary</th>
<th>p-value</th>
<th>TS in Metastasis compared to Primary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cascinu et al</td>
<td>1999</td>
<td>203</td>
<td>41</td>
<td>Liver, Abdominal</td>
<td>47%</td>
<td>82%</td>
<td>p=0.02</td>
<td>Higher in Metastasis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p=0.02</td>
</tr>
<tr>
<td>Aschele et al</td>
<td>1999</td>
<td>245</td>
<td>48</td>
<td>Liver, Liver+Other, Other</td>
<td>48%</td>
<td>39%</td>
<td>NS</td>
<td>NS difference</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aschele et al</td>
<td>2000</td>
<td>201</td>
<td>27</td>
<td>Liver, LN, Pelvis</td>
<td>56%</td>
<td>50%</td>
<td>NS</td>
<td>70%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lower in Metastasis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p=0.001</td>
</tr>
<tr>
<td>Backus et al</td>
<td>2002</td>
<td>202</td>
<td>14 (8*)</td>
<td>Liver</td>
<td>44%</td>
<td>NE</td>
<td>29%</td>
<td>Higher in Metastasis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p=0.014</td>
</tr>
<tr>
<td>Corsi et al</td>
<td>2002</td>
<td>204</td>
<td>60 (49*)</td>
<td>Liver, Lung</td>
<td>33%</td>
<td>33%</td>
<td>NS</td>
<td>53%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lower in Metastasis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p&lt;0.04</td>
</tr>
<tr>
<td>Aschele et al</td>
<td>2002</td>
<td>244</td>
<td>124</td>
<td>Liver, Liver, Pelvis, Other</td>
<td>45%</td>
<td>71%</td>
<td>p=0.06</td>
<td>52%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NS difference</td>
</tr>
<tr>
<td>Bendardaf et al</td>
<td>2007</td>
<td>359</td>
<td>39</td>
<td>Liver, Brain, Ovary, Mesenteric,</td>
<td>Mean TS index: 1.14</td>
<td>NS</td>
<td>Mean TS index: 1.25</td>
<td>NS difference</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Peritoneal, Multiple</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study II</td>
<td>2008</td>
<td>45</td>
<td>45</td>
<td>Liver, Lung</td>
<td>84%</td>
<td>70%</td>
<td>NS</td>
<td>80%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NS difference</td>
</tr>
</tbody>
</table>

TS; Thymidylate synthase, IHC; Immunohistochemistry, No.; Number, pts; patients, *; Numbers of available corresponding metastases, LN; Lymph nodes, NS; Non-significant p > 0.05, NE; Not evaluated.
In the six reports where TS expression was defined using PCR the results was somewhat more homogenous with four studies describing a non-significant difference in TS between liver metastases and primary tumours [360-363]. In conclusion, the results are contradictory mainly due to different methodologies used to determine TS expression but also because of a limited number of cases in each category of metastatic sites.

Table 11B. TS expression (PCR) in distant metastases compared to matched primary tumours.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Ref</th>
<th>No.of pts</th>
<th>Metastatic site</th>
<th>TS Metastasis</th>
<th>p-value</th>
<th>TS Primary</th>
<th>TS in Metastasis compared to Primary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leichman et al</td>
<td>1997</td>
<td>248</td>
<td>46* Liver</td>
<td>Median TSβ-actin</td>
<td>Liver:2.75, Peritoneal:4.7</td>
<td>NS</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>Gorlick et al</td>
<td>1998</td>
<td>208</td>
<td>34* Liver</td>
<td>Mean TSβ-actin</td>
<td>Liver:4.7, Peritoneal:19.7</td>
<td>p&lt;0.001</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>Yamada et al</td>
<td>2001</td>
<td>206</td>
<td>90 Liver</td>
<td>Median mRNA ratio</td>
<td>Liver:0.70, Peritoneal:3.42</td>
<td>p&lt;0.001</td>
<td>Median mRNA ratio:0.98</td>
<td>Higher in Lungmet p&lt;0.001 Lower in Livermet p&lt;0.05</td>
</tr>
<tr>
<td>Etienne et al</td>
<td>2002</td>
<td>205</td>
<td>103 Liver</td>
<td>TS activity Liver:325</td>
<td>fmol/min/mg protein</td>
<td>NE</td>
<td>TS activity:150 fmol/min/mg protein</td>
<td>Lower in Metastasis p=0.037</td>
</tr>
<tr>
<td>Inokuchi et al</td>
<td>2004</td>
<td>360</td>
<td>23 Liver</td>
<td>TSβ-actin Liver:0.20</td>
<td>NE</td>
<td>TSβ-actin:0.16</td>
<td>NS difference</td>
<td></td>
</tr>
<tr>
<td>Kuramochi et al</td>
<td>2006</td>
<td>362</td>
<td>31 Liver</td>
<td>TSmRNA:1.48</td>
<td>NE</td>
<td>TSmRNA:1.43</td>
<td>NS difference</td>
<td></td>
</tr>
<tr>
<td>Sameshima et al</td>
<td>2008</td>
<td>363</td>
<td>43 Liver</td>
<td>TSmRNA:3.98</td>
<td>NE</td>
<td>TSmRNA:3.19</td>
<td>NS difference</td>
<td></td>
</tr>
<tr>
<td>Kobayashi et al</td>
<td>2008</td>
<td>361</td>
<td>31 Liver</td>
<td>TSmRNA:1.59-2.04</td>
<td>NE</td>
<td>TSmRNA:1.52-1.74</td>
<td>NS difference</td>
<td></td>
</tr>
</tbody>
</table>

TS;Thymidylate synthase, PCR;Polymerase Chain Reaction, No.;Number, pts;patients, *;No matched primary tumours available for correlation analysis, LN;Lymph nodes, NS;Non-significant p>0.05, NE;Not evaluated.

A significant variation in TS expression within different metastatic lesions from the same patients has also been reported in three studies, (Table 11 A-B), [203,206,208]. Two studies demonstrated a significantly higher TS level in pulmonary metastases compared with hepatic metastases [206,208]. Another study reported a significantly higher expression of TS in abdominal masses compared to liver metastases [203]. Finally, the last report found a higher TS β-actin mRNA ratio in lymph node metastases and peritoneal metastases than in liver metastases, but this difference was not significant [248]. These observations could have important clinical implications as there are CRC patients with a response to 5-FU-based chemotherapy in their liver metastases, while a continuous progress at the same time is seen in their lung metastases or vice versa.
In this study there was a tendency to a higher TS expression in liver metastases 32/38 (84%) than in lung metastases 7/10 (70%), but this difference was not significant. When analyzing data from 497 CRC patients included in randomized clinical trials, Assersohn et al. found a higher response rate to 5-FU-based chemotherapy in liver metastases (40%) than in pulmonary metastases (21%) and lymph node metastases (18%) respectively [364]. As another study had described a higher TS expression in lung metastases compared to liver metastases Assersohn et al. concluded that this might explain the lower response rate for 5-FU found in lung metastases [208].

The reason for a variable TS expression in metastases compared to their matched primary tumours as well as between different metastatic sites remains unclear. One explanation might be heterogeneity in the expression of TS among patients with CRC. If TS is regulated based on the cell proliferation rate at a particular tumour site this could possibly explain the limited duration of response to 5-FU in metastatic CRC [202]. Another reason could be differing activity levels of transcription factors for example E2F-1 that control TS expression [197]. One study has observed a higher expression of E2F-1 in lung metastases compared with liver metastases [207].

A few studies have evaluated if TS expression in the primary tumour can predict response to 5-FU in metastatic disease, but the results are conflicting. In three of these studies that included more than a hundred patients TS expression in the primary tumour failed to predict 5-FU response in the metastatic setting, (Table 5B) [252,253,255,258]. This is of course unfortunate as many of the patients with advanced CRC have metastases that are inaccessible for biopsy, whereas the primary tumour is more easily approachable.

The majority of studies, that have revealed TS expression in metastases as a predictor of response to 5-FU-based chemotherapy have been non-randomized and small with less than a hundred patients included, (Table 5B). Additional larger and preferably prospective studies are needed. It also has to be further evaluated if TS should be measured separately in each metastatic site in order to predict 5-FU responsiveness in metastatic CRC.

During the last decade we have experienced an increase in the number of patients with advanced CRC, who are potentially candidates for curative resections of liver-and lung metastases. Assessment of TS expression in CRC metastases might be considered as a useful tool to define which of these patients who are most likely to benefit from adjuvant 5-FU-based chemotherapy after metastasectomy. TS determination on liver or lung biopsies from metastases could perhaps also be used to tailor the neoadjuvant chemotherapy for each individual patient before metastatic surgery.

This study indicates that TS expression in liver metastases and lung metastases does not always reflect the expression of TS in the corresponding primary tumours.
When we try to find the optimal treatment for patients with metastatic CRC it might therefore not be fully sufficient to only use TS expression in the primary tumour as a predictive marker for 5-FU-based chemotherapy. Today, non-targeted TS agents such as oxaliplatin and irinotecan as well as antibodies against EGFR and VEGFR are available in the treatment of metastatic CRC. These new treatment options highlight the need for tumour markers that can help us to identify those CRC patients with metastases who are unlikely to respond to TS inhibitors.

Although, we did not find a significant difference in TS expression between liver metastases and lung metastases there was a numerical discrepancy with a higher TS expression in liver metastases. The presence of heterogeneity in TS expression between different metastatic lesions is an important observation that we can learn from in the future development of new prognostic and predictive markers in metastatic CRC.

8.3 Study III

The purpose of this study was to evaluate the role of TS expression in the primary tumour as a prognostic marker in patients with stage II and III CRC with a longer follow-up time (120 months) in an enlarged group where we added another 527 patients to the 862 patients that have previously been reported (527+862=1389) [227]. We also wanted to investigate whether TS expression is a predictive factor in adjuvant therapy according to the administered dose of 5-FU.

Different methods are used to quantify TS expression such as IHC and PCR as there is still no consensus regarding the best way to measure TS despite an extensive research in the field. [223,365]. In this study we used IHC to determine TS expression. In the 2006 ASCO tumour marker guidelines it is concluded that RTPCR even if used in several studies may not be the preferred method to use as determination of TSmRNA levels might not be adequate surrogates for TS enzyme activity [182]. As the majority of the reports in the literature to date have used IHC to define TS expression IHC is preferably recommended a standardization of the IHC technique, but more clear evidence is required regarding the association between TS enzyme activity and TS immunostaining. In our group we have previously demonstrated that TS immunostaining with the monoclonal TS106 antibody reflects the TS enzyme activity in CRC [200].

Various criteria are used to grade TS expression and to determine cut-off points in IHC analyses. Most studies have used four intensity grades in which grade TS 0-1 has been classified as low TS and grade TS 2-3 has been classified as high TS [220]. TS evaluation with IHC will always be semi-quantitative, where the main difficulty is to separate low TS from high TS. Based on the results from our previous report with 862 patients where TS expression was mainly predictive for high TS (grade 2-3) we
wanted to make the analyses more stringent and focus on tumours with the highest TS grade (grade 3). In this current study, we have therefore added another categorization where the intensity of TS expression also is classified as low for TS grade 0-2 and as high for TS grade 3, which has been described earlier by Allegra et al. [228]. Furthermore, as TS is known to be heterogenically expressed in CRC we have analyzed two different areas from each primary tumour with the intention to reduce the effect of heterogeneity.

In this study, TS expression was not a prognostic factor for OS or DFS in the entire group of patients (n=1389), but patients in the surgery alone group (n=718) with TS expression grade 0-1 had a significant longer OS and DFS compared to TS grade 2-3, (p=0.045). When using the classification of TS expression 0-2 versus 3, TS had a stronger prognostic value compared to the classification of TS expression in the category 0-1 versus 2-3, (p=0.002).

This is in contrast to the results from our previous study including 862 of these 1389 patients, where the entire group of patients whose tumors expressed a low TS (grade 0-1) had a longer OS (p=0.04) [227]. In this previous study (n=862) TS expression was also an independent prognostic factor for DFS and OS in the subgroup of patients treated with surgery alone.

To date seven reports using IHC have shown that TS expression is a prognostic marker in CRC, where a low TS correlated with an improved survival, (Table 12), [225,227,228,230,239,366,367]. Another eight reports failed to prove any prognostic value with TS expression assessed with IHC [226,237,238,240,241,243,368,369].
Our finding that TS expression is of no prognostic value in the group of patients treated with adjuvant chemotherapy is in accordance with the results from Aguiar et al. [236]. In a meta-analysis from 2004 including both the adjuvant and advanced disease settings (n=3497), Popat et al. [220] concluded that tumors expressing high levels of TS have poorer OS compared to tumors expressing low levels. In the stage II and III patients (n=2610), where data were available for pooling, a high TS expression only seemed to predict poorer OS for CRC patients treated by surgery alone, which is in agreement with the results from our study.

In our present study (n=1389), the trend of benefit of 5-FU based chemotherapy is more obvious among patients whose tumors express a TS level grade 2-3 than in our previous report (n=862) [227]. The benefit of 5-FU-based chemotherapy was significant in the group of 460 patients with the highest TS expression (grade 3), (p=0.005).

The observation that patients with high TS expression benefit more from adjuvant 5-FU-based chemotherapy is in accordance with the majority of studies in the literature, (Table 5A), [219,225,230,236-239]. No differences in OS or DFS in relation to TS expression were found when comparing patients treated with chemotherapy...
who received $\geq 90\%$ of the planned 5-FU dose with those patients receiving $<90\%$ of the planned 5-FU dose.

In this enlarged study ($n=1389$), with a longer follow-up (120 months) we could not confirm any deleterious effect of adjuvant chemotherapy in patients with low intratumoral TS expression (TS grade 0-1), which we found in our previous report with 862 patients.

We revealed that TS expression, retrospectively assessed with IHC, is an independent prognostic factor in CRC patients treated with surgery alone. We also found a significant benefit of adjuvant 5-FU-based chemotherapy in the subgroup of patients with the highest TS expression (grade 3). Whether the dose of 5-FU was more or less than 90\% of the planned dose did not influence this finding.

Taken together, there is not yet any accepted golden standard for the determination of TS expression. In order to identify the true value of TS expression as a prognostic and predictive marker in CRC there is a need of prospective clinical trials, where standardized unbiased methods for assessment and evaluation of TS are used.

8.4 Study IV

The aim of this study was to analyze MMR protein expression using IHC in patients with stage II and III CRC in a large sample size ($n=1006$) collected under clinical trial conditions.

Among the 1006 patients who were analysed with respect to MLH1 and MSH2, 157 patients (15.6\%) showed a complete loss of protein expression. One hundred and thirty nine patients (13.8\%) had MLH1 negative tumours and 15 (1.5\%) demonstrated a total loss of the MSH2 protein. Only three patients lacked protein expression of both MLH1 and MSH2. MMR-protein expression was a prognostic marker for the entire study population with a significant better median OS in patients with MMR-protein negative tumours, compared to patients with MMR protein positive tumours, ($p=0.01$). MMR protein expression was also significantly associated with OS as well as DFS in multivariate analysis adjusted for gender, age, grade of differentiation, stage of disease and numbers of analyzed lymph nodes, (HR, 0.70 [95\% CI, 0.40 to 0.99]; $p=0.01$).

Furthermore, the prognostic value of MMR protein expression was significant in the subgroup of patients with colon cancer ($n=718$), ($p=0.004$). The survival advantage was especially pronounced in patients with MMR-protein negative tumours localized in the right or transverse colon, ($p< 0.001$).

It is not surprising that MMR protein expression has a stronger prognostic value in the colon and especially in the proximal colon as MSI is a very rare phenomenon in the distal parts of the colon as well as in the rectum [75,76]. One can therefore argue about the rationale of analyzing MSI in rectal cancer patients as they represent
such small proportion of MSI tumours and the interpretation of the results in study IV could have been strengthened and cleaner by limiting the cohort to colon only. The inclusion of rectal cancer cases also introduces confounding factors related to the associated differences in prognosis, local recurrence risk, adjuvant management and the use of radiotherapy in rectum versus colon.

In stage II CRC (n=488), the MMR-status showed a trend towards a prognostic value (p=0.07), whereas the MMR-status was a significant prognostic marker in stage II colon cancer (n=356), (p=0.05). However, MMR protein expression did not yield any prognostic value in stage III CRC (n=518) or stage III colon cancer (n=362).

The results from the survival analyses might have been affected by an inadequate staging as the cancer staging procedure when the Nordic adjuvant trials took place in 1991-1996 was less accurate. In particular, it is uncertain how many patients who truly were stage II.

In the 764/1006 patients (76%), where data on lymph node status was available only 87 patients (11%) had according to the current guidelines more than 12 lymph nodes analyzed [116,117]. The median number of lymph nodes found in the surgical specimens in study IV was 5 (range 0-32). In analysis of survival among the 764 patients with a defined lymph node status MMR-protein expression was still a significant prognostic marker.

The relationship between MSI tumours and a better survival has been demonstrated in a systematic review [287]. However, in this systematic review only 13/32 (41%) of the contributing studies described patients with stage II and III CRC. After the meta-analysis was published in 2005, five additional studies have shown an improved survival among patients with MMR deficient tumours [87,96,288,289, Study IV] (Table 13). According to the literature the prognostic utility of MMR status seems quite clear. This study together with the study by Tejpar et al. are the only studies including randomized cohorts of stage II and III patients revealing that MMR deficiency is correlated to an improved OS and DFS.

**Table 13.** MMR status as a prognostic marker in colorectal cancer 2005 to 2010.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Ref.</th>
<th>No. of pts</th>
<th>Stage</th>
<th>Lesion tested</th>
<th>Method</th>
<th>% MSI-H /MMRP positive</th>
<th>Adjuvant treatment</th>
<th>Longer OS</th>
<th>Longer DFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benatti et al</td>
<td>2005</td>
<td>96</td>
<td>1263</td>
<td>Stage I-IV</td>
<td>CRC</td>
<td>PCR</td>
<td>20.3%</td>
<td>S-FU-based</td>
<td>NE</td>
<td></td>
</tr>
<tr>
<td>Lanza et al</td>
<td>2006</td>
<td>288</td>
<td>718</td>
<td>Stage II-III</td>
<td>CRC</td>
<td>HIC/PCR*</td>
<td>15.9%</td>
<td>S-FU-based</td>
<td>NE</td>
<td>MMRP negative</td>
</tr>
<tr>
<td>Malesci et al</td>
<td>2007</td>
<td>87</td>
<td>893</td>
<td>Stage I-IV</td>
<td>CRC</td>
<td>PCR</td>
<td>10%</td>
<td>S-FU-based</td>
<td>NS</td>
<td>MMRP negative</td>
</tr>
<tr>
<td>Jover et al</td>
<td>2006, 2009</td>
<td>302, 303</td>
<td>754</td>
<td>Stage II-III</td>
<td>CRC</td>
<td>HIC+PCR</td>
<td>10.1%</td>
<td>S-FU-based</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Tejpar et al</td>
<td>2009</td>
<td>289</td>
<td>1327</td>
<td>Stage II-III</td>
<td>Colon ca</td>
<td>PCR</td>
<td>14.2%</td>
<td>S-FU-based</td>
<td>MMRP negative</td>
<td>MMRP negative</td>
</tr>
<tr>
<td>Study IV</td>
<td>2010</td>
<td>1006</td>
<td></td>
<td>Stage II-III</td>
<td>CRC</td>
<td>IHC</td>
<td>15.6%</td>
<td>S-FU-based</td>
<td>MMRP negative</td>
<td>MMRP negative</td>
</tr>
</tbody>
</table>

MMR;Mismatch repair, No.;Number, pts;patients, CRC;Colorectal cancer, PCR;Polymerase chain reaction, IHC;Immunohistochemistry, *;Limited number of patients, MSI-H;Microsatellite instability High, MMRP;Mismatch repair protein, 5-FU;5-Fluorouracil, OS;Overall survival, NE;Not evaluated, DFS;Disease-free survival.
In our study, MMR protein expression did not predict clinical outcome of adjuvant 5-FU with respect to OS or DFS. As the current controversy lies in the predictive utility of MMR status for patients receiving adjuvant 5-FU-based chemotherapy as described in Table 6A it is important to discuss this negative finding in detail. The 1006 patients in study IV represent a group of Swedish patients of a total cohort of 2224 stage II and III CRC patients enrolled in Nordic adjuvant trials, where an absence of a significant survival benefit from adjuvant chemotherapy was reported for stage III disease [342].

Furthermore, the effect of adjuvant chemotherapy is a result of the accumulated dose of chemotherapy administered to each patient and in study IV only 225/491 (46%) of the patients treated with adjuvant chemotherapy received more than 90% of the planned 5-FU dose. It is also important to consider the time interval until start of adjuvant chemotherapy, where a delay longer than 8 weeks has been reported to be negative [370]. The median time to initiation of adjuvant treatment in the Nordic trials was 7 weeks (range 2-23), which is longer than in any of the previous reported randomized adjuvant trials showing a survival benefit of 5-FU [342].

Early clinical trials indicated that 5-FU was beneficial for CRC patients with MSI tumours [300, 301], but these findings are in contrary with the results from in vitro studies showing that CRC cells with MSI are less responsive to 5-FU [371]. Thereafter, a total of five reports [96,297-299,303] have been published addressing the predictive value of MSI in the adjuvant 5-FU treatment of CRC with a better response rate in MMR competent tumours. A nother two studies did not find MMR status to be predictive for response to adjuvant 5-FU [304,305]. T wo of the five reports supporting a survival benefit of adjuvant 5-FU in patients with MMR competent tumours enrolled patients from randomized clinical trials [297,299], one study was prospective [303] while two reports were non-randomized [96,298]. In summary, the value of these results has to be proven in large high-powered prospective trials.

In this study, MMR protein expression was a prognostic marker in the entire study population with a significantly improved OS and DFS among CRC patients with MMR deficient tumours. The prognostic value was even more pronounced when analyzing tumours from the colon and especially from tumours proximal to the splenic flexure.

The importance of the results from this study have to be underlined as we analyzed tumours from a large study cohort (n=1006) with solely stage II and III CRC patients enrolled in randomized adjuvant trials.

These results also support the changes made in the 7th edition of the AJCC Cancer Staging Manual, where AJCC recommends a routine assessment of MSI as a new prognostic factor in colon cancer [115].
8.5 Study V

The primary aim of this study was to investigate if a combined analysis of MMR protein expression and TS expression in colon cancer has a prognostic value and can predict response to adjuvant 5-FU-based chemotherapy. The secondary aim was to evaluate the potential relationship between MMR protein expression and TS expression.

In order to strengthen the evaluation of the prognostic and predictive role of MMR protein expression and TS expression we decided to focus on the subgroup of Swedish patients with colon cancer (n=716), where tumour tissue was available for analysis, out of the 2224 stage II and III CRC patients enrolled onto the Nordic trials randomly assigned to surgery alone or surgery plus adjuvant 5-FU-based chemotherapy [342]. Including rectal cancer cases would complicate the interpretation of the results as there is less knowledge regarding the benefits of adjuvant chemotherapy in rectal cancer [154] and that the rectal location represents a very small portion of MMR deficient tumours [75,76]. The categorization of low TS expression defined as grade 0-2 and of high TS expression defined as grade 3 has been previously reported by Allegra et al. [228] and was used due to its stronger predictive value in study III compared to the traditionally used classification system of low TS (grade 0-1) versus high TS (grade 2-3). Among the 716 patients with colon cancer 142 (20%) showed a loss of MMR protein expression and 273/716 (38%) expressed a high TS (grade 3). In order to perform a combined analysis of MMR protein expression and TS expression the patients were divided into 4 different groups, (Table 14).

Table 14. TS expression (0-2 vs. 3) stratified by MMR protein expression.

<table>
<thead>
<tr>
<th></th>
<th>MMR protein negative</th>
<th>MMR protein positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TS Grade 0-2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1: n=84</td>
<td>12%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2: n=359</td>
<td>50%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>n=443 (62%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TS Grade 3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 3: n=58</td>
<td>8%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 4: n=215</td>
<td>30%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>n=273 (38%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>n=142 (20%)</td>
<td>n=574 (80%)</td>
<td>716</td>
</tr>
</tbody>
</table>

Whilst the number of single marker studies on CRC prognosis and response to adjuvant 5-FU-based chemotherapy has increased dramatically in recent years a combined analysis of several potential markers has been the subject of very few studies.

A combined analysis of MMR protein expression and TS expression has yielded a special interest in predicting response to adjuvant 5-FU in colon cancer as MMR deficient tumour cells are less responsive to 5-FU in vitro [371], and that the main
Results and discussion

The mechanism of 5-FU involves an irreversible inhibition of TS [132]. One hypothesis has therefore been that the expression of TS is different in MMR deficient tumours compared to MMR competent tumours.

In a systematic review as well as in study IV the MMR status was a prognostic factor in CRC, where patients with MMR deficient tumours had an improved prognosis [287]. In another systematic review and meta-analysis (n=3497) TS was defined as a prognostic marker with a better clinical outcome in stage I-IV CRC patients whose tumours express a low TS [220]. Results from the majority of retrospective studies in stage II and III CRC patients evaluating the role of MMR status or TS expression to predict response to adjuvant 5-FU suggest a better treatment effect among patients whose tumours are MMR protein competent [96, 297-299, 303] or have a high TS expression [219, 225, 230, 236, 237, 239, Study III].

Based on the current opinion in the literature as discussed above we have focused on the results from group 1 (patients with MMR protein negative tumours with a low TS, grade 0-2) and group 4 (patients with MMR positive tumours expressing high TS, grade 3).

In group 1 (patients with MMR protein negative tumours with low TS) there was no significant difference in OS according to stage of disease (stage II and III) or treatment. In group 4 (patients with MMR protein positive tumours expressing high TS) we found a significant improved survival in patients with stage III disease who were treated with adjuvant 5-FU-based chemotherapy compared to patients treated with surgery alone (p=0.01). However, there was no significant survival benefit of adjuvant 5-FU in the entire group 4 or among group 4 patients with stage II disease. When comparing group 1 and group 4 there was a trend towards a better survival for patients in group 1, (p=0.09) and in patients treated with surgery alone the OS difference almost reached significance, (p=0.06).

It is hard to compare the results from studies describing a combined analysis of MMR protein expression and TS expression as various methods (IHC and PCR polymerase chain reaction) have been used to quantify MMR status and TS in different tumour locations (colon and rectum) and treatment settings (adjuvant setting and metastatic setting), [372-379], (Table 15).
Table 15. The collected literature of a combined analysis of MMR protein expression and TS expression in colorectal cancer.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>ref</th>
<th>No. of pts</th>
<th>Primary tumour</th>
<th>Stage of disease</th>
<th>Method of MMR status</th>
<th>Method of TS</th>
<th>% MSI-H/MMRP neg</th>
<th>% TS High</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calascibetta et al</td>
<td>2004</td>
<td>373</td>
<td>80</td>
<td>CRC</td>
<td>?</td>
<td>PCR</td>
<td>IHC+PCR</td>
<td>MSI:60%</td>
<td>72%</td>
<td>No</td>
</tr>
<tr>
<td>Ricciardiello et al</td>
<td>2005</td>
<td>378</td>
<td>192</td>
<td>CRC</td>
<td>II-IV</td>
<td>IHC+PCR</td>
<td>IHC</td>
<td>MSI-H:18%</td>
<td>21%</td>
<td>MSI-H correlates with high TS (p&lt;0.001)</td>
</tr>
<tr>
<td>Popat et al</td>
<td>2006</td>
<td>377</td>
<td>441</td>
<td>CRC</td>
<td>I-III</td>
<td>PCR</td>
<td>IHC</td>
<td>MSI:15%</td>
<td>59%</td>
<td>No</td>
</tr>
<tr>
<td>Sinicrope et al</td>
<td>2006</td>
<td>379</td>
<td>320</td>
<td>Colon</td>
<td>II-III</td>
<td>IHC+PCR</td>
<td>IHC</td>
<td>MSI-H:19%</td>
<td>75%</td>
<td>No</td>
</tr>
<tr>
<td>Odin et al</td>
<td>2007</td>
<td>376</td>
<td>181</td>
<td>CRC</td>
<td>I-IV</td>
<td>PCR</td>
<td>PCR</td>
<td>MSI-H: 19%</td>
<td>NE</td>
<td>MSI-H correlates with high TS (p&lt;0.0001)</td>
</tr>
<tr>
<td>Bendardaf et al</td>
<td>2008</td>
<td>372</td>
<td>73</td>
<td>CRC</td>
<td>IV</td>
<td>IHC</td>
<td>IHC</td>
<td>NE</td>
<td>NE</td>
<td>MMMRP neg correlates with low TS (p=0.0001)</td>
</tr>
<tr>
<td>Jensen et al</td>
<td>2008</td>
<td>374</td>
<td>130</td>
<td>CRC</td>
<td>I-IV</td>
<td>PCR</td>
<td>IHC</td>
<td>MSI-H:15%</td>
<td>NE</td>
<td>MSI-H correlates with high TS (p&lt;0.001)</td>
</tr>
<tr>
<td>Jensen et al</td>
<td>2009</td>
<td>375</td>
<td>340</td>
<td>CRC</td>
<td>II-IV</td>
<td>IHC+PCR</td>
<td>IHC</td>
<td>MSI:34%</td>
<td>25%</td>
<td>MSI-H correlates with high TS (p&lt;0.001)</td>
</tr>
<tr>
<td>Study V</td>
<td>2010</td>
<td>716</td>
<td>716</td>
<td>Colon</td>
<td>II-III</td>
<td>IHC</td>
<td>IHC</td>
<td>MMMRPneg:20%</td>
<td>74%</td>
<td>No</td>
</tr>
</tbody>
</table>

MMR; Mismatch repair, TS; Thymidylate synthase, No.; Number, pts; patients, CRC; Colorectal cancer, PCR; Polymerase chain reaction, IHC; Immunohistochemistry, MSI-H; Microsatellite instability-high, MMRP; Mismatch repair protein, NE; Not evaluated.

Our study and the study by Sinicrope et al. [379] are the only studies that exclusively analyzed tumours from patients with stage II-III colon cancer collected from randomized clinical trials. In these two studies no correlation between MMR status and TS expression was found. In two other studies, they also failed to show a correlation between MSI and expression of TS [373,377]. One study, including solely patients with stage IV CRC reported that MSI was correlated to a low TS expression [372]. In the other four studies including CRC patients there was a significant association between MSI and a high expression of TS [374-376,378]. In two of these studies they suggest that this association might explain why MMR deficient tumours often do not respond to 5-FU-based chemotherapy [376,378]. However, in the largest of the four studies (n=340) they showed no evidence that the expression of TS may account for an improved outcome or 5-FU resistance in CRC with MSI [375]. Instead they suggest that tumour biology is more likely the reason for a favourable outcome in MMR deficient CRC. Moreover, all four studies included patients with metastatic CRC, whereas the analysis of TS was only performed on the primary tumour [374-376,378]. It is important to point out that TS expression has been a more convincing predictive marker in disseminated disease, where most studies have shown that a high TS expression in the metastases is associated with lack of response to 5-FU (Table 5B) [203,229,233-235,244-250]. This is in contrary to the adjuvant setting as already
mentioned, where a high TS expression instead has been associated with an improved response to 5-FU (Table 5A) [219,225,230,236,237,239, Study III].

In this study we have indicated that a combined analysis of MMR status and TS expression might add valuable information, where stage III colon cancer patients with MMR protein positive tumours expressing a high TS seem to gain most from adjuvant 5-FU.

We could not find any significant correlation between MMR protein expression and TS expression in study V and therefore TS expression alone cannot explain the resistance to 5-FU in MMR deficient tumours.
9 SUMMARY AND CONCLUSION

The objectives of the studies in this thesis were to compare the expression of TS in lymph node metastases as well as liver metastases and lung metastases of CRC with TS expression in their matched primary tumours and to evaluate the prognostic and predictive role of thymidylate synthase expression and mismatch repair protein expression in stage II and III CRC.

The results from this thesis can be summarized as follows:

- TS expression in lymph node metastases and their corresponding primary tumours was assessed in 348 patients with Dukes’C CRC. A high expression of TS was found in 67% of the lymph node metastases compared to 74% of the matched primary tumours. There was a significant correlation between the expression of TS in lymph node metastases and TS in primary tumours. TS in lymph node metastases was a prognostic factor in the entire study group with regard to OS and DFS, where a low TS expression correlated with a longer OS and DFS. TS expression in lymph node metastases also had a significant prognostic value in multivariate analysis with respect to OS and DFS, independently of age, gender, site of tumour and treatment. In subgroup analysis, TS in lymph node metastases was a significant prognostic marker in patients treated with surgery alone for OS and DFS, but not in patients who received adjuvant chemotherapy. TS expression in the primary tumour only had a significant prognostic value among patients, who were treated with surgery alone.

- TS expression in liver metastases (n=38) and lung metastases (n=10) and the expression of TS in their corresponding primary tumours (n=45) was analyzed in CRC patients, who underwent metastatic surgery. A high TS expression was seen in 81% of the distant metastases and in 80% of the primary tumours. Among the 39 patients with a high TS expression in their distant metastases 79% also had a high TS expression in their primary tumours. There was no significant correlation between TS expression in distant metastases and their matched primary tumours. There was a tendency to a higher TS expression in liver metastases (84%) than in lung metastases (70%), but this difference was not significant.

- TS expression retrospectively assessed with IHC was not a prognostic marker for OS or DFS in the entire group of patients (n=1389). In the subgroup treated with surgery alone (n=718), patients whose tumours expressed low TS (grade 0-1) had a significant longer OS and DFS compared to patients with high TS expression (grade 2-3) in their tumours. When using the alternative classification
of TS expression 0-2 as low TS versus 3 as high TS, the prognostic value of TS was stronger. There was a trend towards a benefit of adjuvant 5-FU based chemotherapy in patients whose tumors express TS grade 2-3 (n=990). However, the benefit of adjuvant 5-FU-based chemotherapy was significant in the group of patients with the highest TS expression, grade 3, (n=460). No differences in OS or DFS in relation to TS expression were found when comparing patients treated with chemotherapy, who received $\geq 90\%$ of the planned 5-FU dose with those receiving $< 90\%$ of the planned 5-FU dose. In this enlarged study (n=1389) with a longer follow-up (120 months) we could not confirm any deleterious effect of adjuvant chemotherapy in patients with low TS expression (TS grade 0-1), which was found in our previous report with 862 patients [227].

• In a large cohort of randomized patients (n=1006) with stage II and III CRC 157 (15.6\%) patients showed a loss of MMR protein expression. We found MMR protein expression to be an independent prognostic marker for OS and DFS in univariate analysis as well as in multivariate analysis adjusted for gender, age, grade of differentiation, stage of disease and numbers of analyzed lymph nodes. Patients with MMR protein negative tumours had an improved survival compared to patients with MMR protein positive tumours. The prognostic value of MMR protein expression was also significant in the subgroup of patients with colon cancer, where the survival advantage was especially pronounced in patients with tumours localized proximal to the splenic flexure. We could not demonstrate that MMR protein expression predicts outcome of adjuvant 5-FU with respect to OS or DFS.

• In a combined analysis of MMR protein expression and TS expression in colon cancer there was no significant difference in OS and DFS according to stage of disease (stage II and III) or treatment (surgery alone versus surgery plus adjuvant 5-FU-based chemotherapy) in group 1 (patients with MMR protein negative tumours with low TS). In group 4 (patients with MMR protein positive tumours expressing high TS) we found a significant improved survival in patients with stage III disease, who were treated with adjuvant 5-FU-based chemotherapy compared to patients treated with surgery alone. There was no significant survival benefit of adjuvant 5-FU in the entire group 4 or among group 4 patients with stage II disease. We could not find any significant correlation between MMR protein expression and TS expression indicating that TS expression alone cannot explain the resistance to 5-FU in MMR deficient tumours.
In conclusion data from this thesis indicates that:

- TS expression assessed in lymph node metastases of CRC improves the prognostic precision compared with TS expression defined in the primary tumour but cannot predict response to 5-FU-based adjuvant chemotherapy. A low TS expression in lymph node metastases correlates to a longer OS and DFS.

- TS expression in liver metastases and lung metastases not always reflect the expression of TS in their matched primary tumour.

- TS expression is a prognostic marker in patients treated with surgery alone, where a better survival was seen in patients expressing low TS. Patients with the highest TS expression (grade 3) have a significantly longer OS when treated with adjuvant 5-FU-based chemotherapy, but whether the dose of 5-FU was more or less than 90% of the planned dose did not influence this finding.

- MMR protein expression is a prognostic marker in stage II and III CRC with an improved survival in patients with MMR protein negative tumours, but did not predict response to adjuvant 5-FU-based chemotherapy.

- A combined analysis of MMR protein expression and TS expression might improve the prediction of response to 5-FU-based chemotherapy in stage III patients with MMR protein positive colon tumours expressing high TS. No significant correlation between MMR protein expression and TS expression was found.
10 FUTURE PERSPECTIVES

10.1 Prognostic and predictive markers

Thymidylate synthase
- It would be of great value to retrospectively evaluate TS expression in lymph node metastases as a prognostic and predictive marker in stage III CRC with an accurate number of lymph nodes (12 or more) or a sentinel node available for analysis as described by Troche et al. [357].
- In order to make the prediction of response to adjuvant 5-FU in CRC more stringent, it could be interesting to further investigate TS grade 3 as a predictor in a prospective adjuvant trial. It could also be of interest to test if TS grade 3 in lymph node metastases could add valuable predictive information regarding response to adjuvant 5-FU within different subsets of the heterogeneous group of stage III CRC patients.

MMR status
- To better define MMR protein expression as a stage dependent prognostic marker in stage II and III CRC it would be valuable to find out if the results from study IV could be reproduced in an adjuvant trial setting where an adequate (12 or more) number of lymph nodes have been analyzed.
- The patients included in study IV might not have been the best patient selection for evaluating a potential predictive marker of adjuvant 5-FU in CRC as there was an absence of a significant survival benefit of adjuvant 5-FU-based chemotherapy in the entire Nordic study cohort of 2224 patients [342]. The interpretation of the results in study IV could have been strengthened by solely including patients with colon cancer as MSI is uncommon in rectal cancer and the benefit of adjuvant 5-FU in rectal cancer is less clear. Prospective clinical trials exclusively including colon cancer patients, where at least 12 lymph nodes have been analyzed to reassure a correct tumour staging, could help us to further evaluate MMR protein expression as a predictor of benefit from adjuvant chemotherapy.

A combined analysis of different prognostic and predictive markers
There are several potential combinations of prognostic and predictive markers worth testing in CRC as a single marker might not gain enough sensitivity and specificity.
Study V indicated that a combined analysis of MMR protein expression and TS expression can predict response to adjuvant 5-FU-based chemotherapy in stage III colon cancer, with a better response among patients with MMR positive tumours expressing high TS. It would be of interest to retrospectively perform a combined analysis of MMR protein expression and TS expression in different stage III subsets of patients with colon cancer as stage III patients represent a heterogeneous group with various risk levels for tumour recurrence.

Oxaliplatin together with 5-FU is the accepted standard for adjuvant treatment of stage III colon cancer. Therefore, other tumour markers have to be added to the combination used in study V or we need to identify new combinations of tumour markers that better can predict the treatment effect of 5-FU and oxaliplatin. The MMR status is not involved in resistance to oxaliplatin, even if it is a potential resistance mechanism of other platinum drugs [295]. Oxaliplatin is a third-generation platinum analogue that causes DNA-damaging cross-links blocking DNA replication as well as transcription and initiates apoptosis [144]. Excision cross-complementing gene (ERCC1) is an excision nuclease that has a major role in repairing platinum-induced DNA-adducts. A low expression of ERCC1 has been associated with a better OS in patients with advanced CRC treated with oxaliplatin based chemotherapy [250]. The role of ERCC1 in the adjuvant setting as a single predictive marker for response to oxaliplatin or in combination with other potential predictive markers such as MMR status and TS expression has to be further investigated.

A recently published report demonstrated that the loss of MMR function may predict an improved clinical outcome in patients treated with adjuvant 5-FU and irinotecan compared to patients only receiving 5-FU [307], which is in accordance with the in vitro findings that MMR deficient CRC cell lines have an increased sensitivity to irinotecan [296]. Irinotecan, an inhibitor of the nuclear enzyme topoisomerase-I (topo-I) [142], has historically failed to prove effect in the adjuvant setting even if widely used in metastatic CRC [143]. However, there might be a subset of patients that in fact has effect of adjuvant irinotecan if reliable predictive markers of response to irinotecan are available. It could be worth testing adjuvant irinotecan in patients with MMR deficient tumours with a low expression of TS in a prospective clinical trial. Furthermore, increased levels of the target enzyme topo-I seem to correlate with response to irinotecan in colon cancer cell lines in vitro [380]. In advanced CRC topo-I has been identified as a predictive marker associated with benefit to either irinotecan or oxaliplatin, and as prognostic marker in patients receiving 5-FU alone [381]. It would be of interest to evaluate the potential use of topo-I as a predictor for response to chemotherapy in the adjuvant setting.
10.2 Classification of colorectal cancer based on molecular, clinical and morphological features

It is most unlikely to find a single prognostic and predictive molecular marker in CRC. We rather have to classify different subtypes of CRC based on the correlation of molecular, clinical and morphological features as suggested by the pathologist Jass in order to identify CRC patients with a poor prognosis, who will truly benefit from therapy, Table 16 [24]. This is particularly important in stage II and III CRC, where some of the patients can be cured by surgery alone. In this classification MMR status as an important molecular feature is a corner-stone. For many decades clinical management as well as research has defined CRC as a homogenous entity instead of a complex heterogeneous multi-pathway disease. CRC within a certain TNM stage might in fact have very different morphological characteristics as well as molecular features that can affect prognosis as well as response to therapy. The changes made in the 7th edition of the AJCC Cancer Staging Manual, where AJCC recommends a routine assessment of MSI as a new prognostic factor in colon cancer is the first move towards a molecular classification of this disease [115].

- There are several clinical and morphological characteristics with prognostic value as described in detail in chapter 3. However, the problem is the lack of accepted standards and guidelines for the pathological evaluation of these morphological prognostic factors. It would be challenging to implement a modified version of the classification made by Jass in a clinical trial setting in order to evaluate a more personalized approach to the treatment of CRC.
Table 16. Molecular, clinical and morphological features of colorectal cancer groups 1–5 [24]

<table>
<thead>
<tr>
<th>Feature</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSI status</td>
<td>H</td>
<td>S/L</td>
<td>S/L</td>
<td>S</td>
<td>H</td>
</tr>
<tr>
<td>Methylation</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>Ploidy</td>
<td>Dip &gt; An</td>
<td>Dip &gt; An</td>
<td>An &gt; Dip</td>
<td>An &gt; Dip</td>
<td>Dip &gt; An</td>
</tr>
<tr>
<td>APC</td>
<td>+/−</td>
<td>+/−</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>KRAS</td>
<td>−</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>BRAF</td>
<td>+++</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>TP53</td>
<td>−</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Location</td>
<td>R &gt; L</td>
<td>R &gt; L</td>
<td>L &gt; R</td>
<td>L &gt; R</td>
<td>R &gt; L</td>
</tr>
<tr>
<td>Gender</td>
<td>F &gt; M</td>
<td>F &gt; M</td>
<td>M &gt; F</td>
<td>M &gt; F</td>
<td>M &gt; F</td>
</tr>
<tr>
<td>Precursor</td>
<td>SP</td>
<td>SP</td>
<td>SP/AD</td>
<td>AD</td>
<td>AD</td>
</tr>
<tr>
<td>Serration</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Mucinous</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Dirty necrosis</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Poor differentiation</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Circumscribed</td>
<td>+++</td>
<td>+</td>
<td>?</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Tumour budding</td>
<td>+/-</td>
<td>+</td>
<td>?</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>+++</td>
<td>+</td>
<td>?</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

MSI, microsatellite instability; H, high; S, stable; L, low; Dip, diploid; An, aneuploid; Serration, serrated morphology; SP, serrated polyp; AD, adenoma; Circumscribed, circumscribed invasive margin


10.3 Metastatic pattern in colorectal cancer

Dissemination and colonization by tumour cells is still a largely unknown complex process with many different steps where gene alterations are involved [382].

- It would be important to investigate why MMR competent primary colorectal tumours have an increased metastatic potential. Data suggests that these tumours have an infiltrating tumour border configuration with tumour budding [383], which could be one explanation. In our group we are currently analyzing tumour budding according to MMR status in CRC using IHC and staining with the anti-cytokeratin antibody MNF-116 [384]. The invasion process with a degradation of
the basal membrane and ECM (extracellular matrix) depends on different proteinases such as matrix metalloproteinases (MMPs) which are produced by cancer cells [385]. Matrilysin (MMP-7) that is overexpressed in most CRCs is correlated with and in increased metastatic capacity [386,387]. It would therefore be of interest to find out if the expression of MMP-7 is higher in MMR competent tumours compared with MMR deficient tumours.

• In study II, we demonstrated that TS expression in liver metastases and lung metastases not always reflect the expression of TS in their matched primary tumour. There are several studies demonstrating that TS expression in the primary tumour can not predict response to 5-FU in metastatic disease. Therefore, it might be necessary in advanced CRC to more frequently analyze prognostic and predictive marker of interest in biopsies from the metastases and not only in the primary tumours. There are an increasing number of patients who will become eligible for curative resection of liver metastases and lung metastases. In this group of patients it might be of importance to further evaluate potential predictive markers in distant metastases in order to identify those patients who will gain the most from adjuvant chemotherapy after metastasectomy.

• In a recent published report evaluating protein expression of MLH1, MSH2 and PMS2 in 92 primary tumours and their corresponding lymph node metastases and liver metastases a differing expression of MLH1/PMS2 between primary tumours and liver metastases was found [266]. They revealed the presence of distinct subclones of MLH1/PMS2 positive cells in the invasive front within seven of eight primary tumours with predominantly MLH1/PMS2 negative cells in the transition zone. These seven primary tumours also showed a MLH1/PMS2 positive expression in their corresponding lymph node metastases and liver metastases. In conclusion they speculate if that could be a result of MLH1 re-expression through promoter de-methylation. If true this result could have implications when comparing chemosensitivity of metastases and matched primary tumours. This study also addresses another important issue regarding which part of the primary tumour that should preferable be analyzed when evaluating new prognostic and predictive markers in CRC; the transition zone or the invasive front?

10.4 Novel methods to use in future analyzes of prognostic and predictive markers

In recent years there has been a rapid development of techniques for gene and protein expression profiling, such as TMA, DNA microarrays, and mass spectrometry,
According to the existing knowledge, a very limited percentage of the genome (1.1%) consists of exons coding for proteins, which is important to be aware of when evaluating the results from genomic and proteomic studies. Protein research can be performed using TMA, but more often proteomics is associated with the use of mass spectrometry. Hopefully, these new techniques will increase our understanding of different CRC subtypes that can help us to yield new prognostic and predictive markers. Another interesting field within cancer molecular biology with great potential is MicroRNAs (miRNA), that has dramatically changed the view on gene regulation.

**TMA**

The TMA technique is especially good at testing if genes found to be differentially expressed in CRCs gene expression profiling are also distinct on the protein level [231]. There are advantages as well as disadvantages with using IHC on TMA compared to traditional IHC methods using whole mounted tumour sections. One advantage is that when using TMA the immunostaining can be performed under identical conditions even with a high number of tissue samples have to be assessed and valuating a single tumour punch might also increase inter-observer agreement. The major disadvantage with TMA is the evaluation of the presence and extent of heterogeneity in protein expression within a carcinoma as the analysed area of tumour tissue is limited.
DNA microarray

The DNA microarray technology allows the analysis of gene expression patterns of thousands of different genes in one tumour sample. The gene signatures that have been identified contain genes that regulate cell proliferation, cell signaling and immune responses. Gene expression profiling has been shown to be clinically useful in predicting the risk of poor outcome in different tumour types. Gene expression profiles as predictors of poor outcome in stage II CRC has recently been described in a systematic review and meta-analysis [389]. Future studies have to investigate whether the different identified gene signatures reflect alteration in the same generic pathways or not. The main focus in gene expression profiling so far has been prognosis, but the next step will be to define gene signatures that can predict response to chemotherapy. When using DNA microarray technology, there are several confounding factors to considerate such as differences in methodology and experimental conditions. Before this method can be implemented in a clinical setting, there is a need for standardization of the procedures in order to facilitate comparative analysis. Gene expression profiling has an enormous potential, but is technically very demanding and requires expensive high quality tissue management and the cost-effectiveness is therefore an important issue to discuss.

MicroRNA (miRNAs)

MicroRNAs (miRNAs) are small non-coding RNAs (18-25 nucleotides) that target mRNAs in order to regulate the translation process and thereby gene expression. Many proteins involved in key signaling pathways of CRC development seem to be affected by miRNAs. Different tissue expression profiles of miRNAs have been associated with prognosis and therapeutic outcomes in CRC [390]. Furthermore, miRNAs seem to be potential novel therapeutic targets [391]. Interestingly, recent data also indicates that miRNAs occur in circulating blood and might be used as an early diagnostic option for CRC [392].
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