# Prognostic role of tumour associated trypsin inhibitor in colorectal cancer patients

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# PROGNOSTIC ROLE OF TUMOUR ASSOCIATED TRYPSIN INHIBITOR IN COLORECTAL CANCER


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# List of papers

## Papers included in the thesis:

I. **Gaber A**, Johansson M, Stenman UH, Hotakainen K, Ponten F, Glimelius B, Bjartell A, Jirstrom K, Birgisson H. High expression of tumour-associated trypsin inhibitor correlates with liver metastasis and poor prognosis in colorectal cancer.

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II. **Gaber A**, Nodin B, Hotakainen K, Nilsson E, Stenman UH, Bjartell A, Birgisson H, Jirström K. Increased serum levels of tumour-associated trypsin inhibitor independently predict a poor prognosis in colorectal cancer patients.

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III. **Gaber A**, Stene C, Hotakainen K, Nodin B, Palmquist I, Bjartell A, Stenman UH, Jeppsson B, Johnson L, Jirström K. Effects of radiation therapy on tissue and serum levels of tumour associated trypsin inhibitor in rectal cancer patients.

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IV. **Gaber A**, Fridberg M, Nodin B, Edsjö A, Hotakainen K, Stenman UH, Bjartell A, Birgisson H, Jirström K. Association of tumour-associated trypsin inhibitor with expression of EGFR signalling pathway proteins, KRAS mutation status and clinical outcome in colorectal cancer.

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# Papers not included in the thesis:

Borgquist S, Djerbi S, Pontén F, Anagnostaki L, Goldman M, **Gaber A**, Manjer J, Landberg G, Jirstrom K. HMG-CoA reductase expression in breast cancer is associated with a less aggressive phenotype and influenced by anthropometric factors.

*Int J Cancer* 2008 Sep 1; 123(5):1146-53.

Dahlman A, Rexhepaj E, Brennan DJ, Gallgher WM, **Gaber A**, Lindgren A, Jirström K, Bjartell A. Evaluation of the prognostic significance of MSMB and CRISP3 in prostate cancer using automated image analysis.

*Modern Pathology* 2011 May; 24(5):708-19

Larsson A, Johansson ME, Wangefjord S, **Gaber A**, Nodin B, Kucharzewska P, Welinder C, Belting M, Eberhard J, Johnsson A, Uhlén M, Jirström K. Overexpression of podocalyxin-like protein is an independent factor of prognosis in colorectal cancer.

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Wangefjord S, Manjer J, **Gaber A**, Nodin B, Eberhard J, Jirström K. Cyclin D1 expression in colorectal cancer is a favourable prognostic factor in men but not women in a prospective, population-based cohort study.

*Biology of Sex Differences* 2011 Sep 2:10

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*Proteomics Clin Appl* 2011 Dec (11-12):624-35

Eberhard J, **Gaber A**, Wangefjord S, Nodin B, Uhlén M, Ericson Lindquist K, Jirström K. A cohort study of the prognostic and treatment predictive value of SATB2 expression in colorectal cancer.

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# Abbreviations

| | |
|---|---|
| 5-FU | 5-fluorouracil |
| AKT | Protein kinase B |
| AUC | Area under the curve |
| *BRAF* | v-RAF murine sarcoma viral oncogene homolog B1 |
| CA | Carbohydrate antigen |
| CEA | Carcinoembryonal antigen |
| CI | Confidence interval |
| CIS | Carcinoma in situ |
| CpG | Cytosine preceding guanine |
| CRC | Colorectal cancer |
| CSS | Cancer specific survival |
| CTC | Circulating tumour cell |
| DAB | 3,3-diaminobenzidine chromogen |
| DFS | Disease free survival |
| ECM | Extracellular matrix |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| ERK | Extracellular signal-regulated kinase |
| FAP | Familial adenomatous polyposis |
| Gy | Gray |
| HIER | Heat induced epitope retrieval |
| HNPCC | Hereditary non polyposis colorectal cancer |
| HR | Hazard ratio |
| IFMA | Time-resolved immunofluorometric assay |
| IHC | Immunohistochemistry |
| *KRAS* | Kirsten rat sarcoma viral oncogene homolog |
| LOH | Loss of heterozygosity |
| M-stage | Metastatic stage |
| mAb | Monoclonal antibody |

| | |
|---|---|
| MAPK | Mitogen-activated protein kinase |
| MMP | Matrix metallo proteinase |
| MMR | Mismatch repair |
| MSI | Micro satellite instability |
| MSS | Micro satellite stable |
| MSP | Matrix serine proteinase |
| N-stage | Nodal stage |
| NSAID | Non steroid anti inflammatory drug |
| OS | Overall survival |
| PAI-1 | Plasminogen activator inhibitor 1 |
| PAR | Protease-activated receptor |
| P13K | Phosphatidylinositol 3 kinase |
| PSTI | Pancreatic secretary trypsin inhibitor |
| RIA | Radioimmunoassay |
| ROC | Receiver operating characteristics |
| RT | Radiotherapy |
| s-TATI | Tumour associated trypsin inhibitor in serum |
| SPINK1 | Serine protease inhibitor Kazal type-1 |
| T-stage | Tumour stage |
| t-TATI | Tumour associated trypsin inhibitor in tumour tissue |
| TGF-ß | Transforming growth factor beta |
| TTR | Time to recurrence |
| TMA | Tissue micro array |
| *TP53* | Tumour gene 53 |
| PTEN | Phosphatase and tensin homolog |
| UC | Ulcerative colitis |
| VEGF | Vascular endothelial growth factor |
| VEGFR | Vascular endothelial growth factor receptor |

# Abstract

Colorectal cancer (CRC) is one of the most common forms of human cancer worldwide with approximately 1 million new cases detected every year. CEA is currently the only accepted CRC marker incorporated into clinical practice, where it is used for early detection of metastasis in follow-up of patients having stage II and III disease, and for monitoring response to adjuvant treatment. However there are no reliable prognostic and response predictive biomarkers for CRC. Tumour-associated trypsin inhibitor (TATI) is a form of antitrypsin, a 6kDa enzyme that inhibits trypsin with great affinity and TATI has previously been found to be of prognostic importance in mucinous ovarian cancer and other cancer forms. TATI is expressed in normal colonic mucosa, where its main function is to act as an antitrypsin, protecting the tissue from proteolysis. TATI has also been shown to promote migration and invasion in colorectal cancer cell lines in vitro. Moreover, TATI has a similar molecular structure to epidermal growth factor (EGF) and has been found to bind to EGFR and promote malignant behaviour in various cell line models. The prognostic significance of TATI in CRC patients had however not been reported and the main aim of the present investigation was therefore to examine levels of TATI in tumour tissue and serum samples from three independent CRC patient cohorts, with particular reference to their association with clinical outcome. In addition, we assessed the effect of neoadjuvant radiation therapy (RT) on TATI levels in tissue and serum of rectal cancer patients, and whether expression of epidermal growth factor receptor (EGFR) and markers of downstream signalling have a modifying effect on the prognostic value of TATI. In paper I, we investigated TATI expression by immunohistochemistry (IHC) in CRC tumours (t-TATI) in two cohorts adding up to 424 patients. High TATI expression was found to be significantly associated to short overall survival (OS), and disease free survival (DFS), as well as an increased risk of liver metastasis. In paper II, we analysed the prognostic value of TATI in serum (s-TATI) in one of the cohorts used in Paper I (n = 334). While no significant correlation was found between s-TATI and t-TATI concentrations, the prognostic value of s-TATI was more evident than for t-TATI, the former being a strong independent predictor of an impaired OS, DFS and time to recurrence (TTR), suggesting that elevated serum concentrations are not a result of TATI production by the tumour. Further results from paper II revealed that s-TATI concentrations were

lower in rectal cancer patients, the majority of whom had received neoadjuvant radiotherapy (RT). In paper III, we therefore ventured to investigate whether neoadjuvant RT affects TATI concentrations in tissue and serum. To this end, we analysed serum and tissue samples collected at multiple timepoints from 53 patients with rectal cancer included in a in a case-control study. The results revealed that t-TATI and s-TATI levels remained unaffected by RT. S-TATI was significantly associated with shorter OS at all timepoints, and t-TATI assessed in surgical specimens was also a factor of poor prognosis. In paper IV we investigated the associations of s-TATI and t-TATI to EGFR expression and downstream signalling proteins pSTAT3 and pERK1/2, as well as *KRAS* mutation status. Apart from a significant association between t-TATI expression and KRAS mutation status, there was no intercorrelation between t-TATI or s-TATI and the investigative markers. Expression of pERK1/2 and pSTAT3, and KRAS mutation status, was significantly associated with poor prognosis. The prognostic value of EGFR expression, being non-significant per se, was modified by s-TATI concentrations in that patients with EGFR positive tumours/ high s-TATI levels had a significantly shorter survival compared to patients with EGFR negative tumours/ low s-TATI levels.

Keywords: TATI, Colorectal cancer, Prognosis, Biomarker, EGFR, STAT3, ERK1/2, *KRAS* mutation

# Background

## Short introduction to colorectal cancer

Colorectal cancer is the third most commonly diagnosed cancer form and approximately 1 million individuals worldwide are diagnosed with the disease each year [1]. Amongst the countries with the highest incidences of CRC are Japan, Australia and New Zeeland [1]. In Sweden, the reported annual incidence of colon cancer between 2003-2007 was 16.6 (per 100.000 inhabitants) in men and 13.9 for women. The corresponding figures for rectal cancer were 9.3 for men, and 6.5 for women [2]. CRC is more frequent in elderly patients [3]. In Sweden and the Nordic countries, with exception for Denmark, CRC patients have high survival rates compared to the rest of Europe [4, 5].

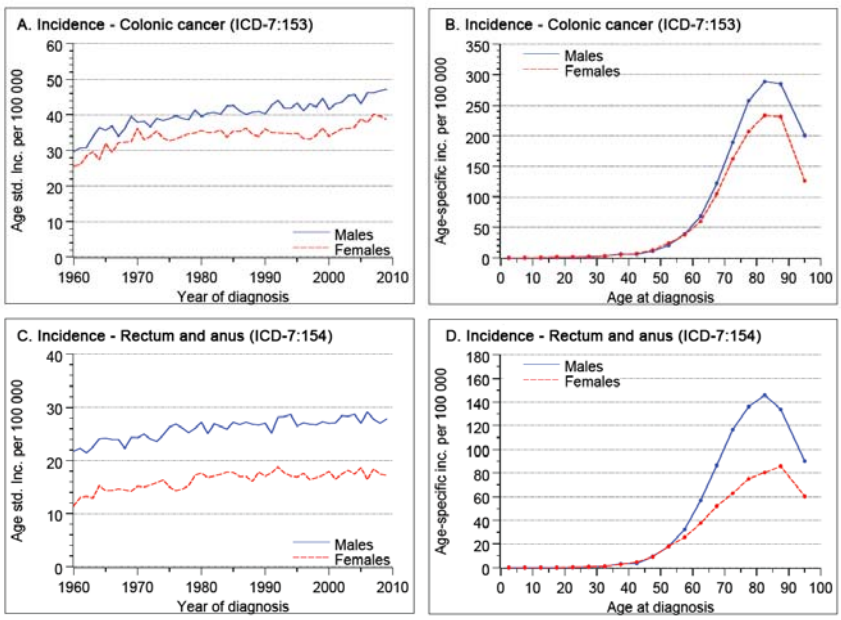


Figure 1. The incidence of colon (A-B) and rectal cancer (C-D), according to the Swedish Cancer Registry. Graphs are reproduced with permission from Socialstyrelsen [6].

Carcinogenesis could be summarised as an event whereby cells accumulate genetic damage and over time acquire the ability to be self sufficient, uncontrollable regarding cell signalling, non-responsive to inhibitory signals and capable to avoid endogenous apoptotic mechanisms [7]. In most cases, CRC has a long incubation time, where the transformation from healthy tissue to cancerous tissue could take as long as 35 years. Therefore the incidence of the disease is much more common in elderly patients [8, 9]. The majority of CRC tumours derive from adenomatous polyps. Studies have shown that it usually takes approximately 10 to 15 years for CRC to develop from an adenomatous polyp and it has been estimated that half of all individuals will eventually develop adenomas in their lifetime [10]. As genetic alterations progrediate in an established CRC, metastatic properties may be acquired. Early studies on metastasis have revealed that only a small percentage, as low as 1% of all tumour cells reaching the circulation, have chance to establish metastasis, while the rest perish [11]. Approximately 30-40% of CRC patients that have non-metastatic disease and are treated with curative intent, will eventually relapse with metastases [review][12]. Different cancer forms metastasize to different organs, and for CRC, the liver is the primary site of metastasis. It was already discovered as early as in the 20th century that tumours tend to metastasize to the first organ they encounter in the hemodynamic system [13]. There have also been early studies showing that tumour cells from a particular organ more frequently metastasize in certain environments [14]. In the case of CRC, this occurs as metastatic cells i.e. circulating tumour cells (CTC) that enter the circulation, travel through the vena porta and encounter the liver. The second most common metastatic site is the lungs [14].

## Risk factors

The aetiology of CRC is debatable, however some of the most common risk factors of developing the disease are high age, inherited genetic mutations environmental factors and inflammatory bowel disease [15-17]. Regarding the latter, a meta-analysis from 2001 showed that ulcerative colitis (UC) is a strong risk factor of CRC, with 2% of all UC patients developing CRC 10 years after their UC diagnosis, and after 20 years the percentage increased to 8% [18]. Patients with Crohn's disease also have an increased risk of CRC, but somewhat lower than patients with UC [15]. Further on, smoking [19, 20], and obesity [21, 22] have also been associated with an increased risk of CRC, the latter particularly in males [21, 23, 24]. Alcohol consumption has in some studies been found to marginally increase the risk of CRC [25] and diet with a high proportion of red meat has been widely debated as a risk factor. In a large study, red meat consumption did not remain significant when adjusted for

confounders including smoking and obesity [26], while in another study, comparing populations of 15 European countries, significant associations to red meat consumption was found in 12 countries for males, but only in 6 countries for females [27]. A recent meta-analysis established the association between CRC and red meat as inconclusive [28]. Other common dietary factors have also been subject to investigation with contradicting results, such as intake of milk protein, vegetables, rye products, processed food and products containing calcium [29].

# Colorectal carcinogenesis

Important genes involved in colorectal carcinogenesis can be divided into three subcategories; oncogenes, tumour suppressor genes and mismatch repair (*MMR*) genes.

*KRAS* is an oncogene encoding the KRAS-protein, which belongs to the RAS super family, and is located at the inner surface of the cell membrane [30, 31], [reviewed in][32]. The KRAS protein could be viewed as an on-off switch for activation of the MAPK-signalling pathway [review ][31, 33]. Mutations of the *KRAS* gene can, by production of mutant protein, cause autosomal activation of downstream MAPK signalling [review][33], independent of extracellular ligand activation. The predominant sites of mutation in the *KRAS* gene are in codons 12, 13 and 61 [34]. Approximately 35-40% of CRC tumours have *KRAS* mutations in codon 12 or 13 [35, 36], and mutations have been found to occur in an early stage of colorectal carcinogenesis [37, 38] as well as in other cancer forms [39]. These types of mutations have been found to be more prevalent in advanced stages of CRC [36]. Studies on the prognostic value of *KRAS* have revealed varying results [40-47], some reporting an association with poor outcome, while others failed to demonstrate any prognostic significance [43, 44, 46]. Another oncogene, which encodes for the B-type Raf kinase (BRAF) protein, has also been shown to activate the MAPK-pathway [48]. *BRAF* mutations have been found in 2-8% of CRC tumours [46, 49] and correlate with poor prognosis [44, 46, 47], but its ability to predict response to anti-EGFR treatment is debated and remains to be further investigated [46]. *KRAS* and *BRAF* mutations have been found to be mutually exclusive [38, 49], while both mutations seem to occur at tumour initiation [38] (Figure 2).

Another gene of importance in colorectal carcinogenesis is the *TP53* gene that encodes the tumour suppressor protein p53, which has been described as the "guardian of the genome" [8, 50], since it plays a central role in the induction of genes that are important in cell cycle arrest and apoptosis following DNA damage

[review][51]. Mutations in the *TP53* gene are present in approximately 45-55% of CRC tumours [52, 53],[review][54], but in contrast to *KRAS* and *BRAF*, *TP53* mutations mostly occur at a later stage of carcinogenesis [55]. TP53 mutations have been associated with poorly differentiated and clinically more aggressive CRC[56] and studies on the prognostic value of *TP53* mutations have revealed an association with an impaired survival [57, 58]. It has also been shown that patients with *TP53* mutated tumours do not benefit from treatment with 5-fluorouracil (5-FU) [54, 59].

Mutation of another tumour suppressor gene, the adenomatous polyposis coli (*APC*) gene, has also been suggested as a key event in CRC carcinogenesis [60]. Approximately 60% of CRC tumours harbour this mutation, which occurs early in colorectal carcinogenesis, being present also in adenomas [61]. Mutations of the *APC* gene have been found to induce polyposis, which is associated with a highly increased risk of CRC [62] but no prognostic value has been found for this mutation [63]. A study on *APC, KRAS* and *TP53* mutations, showed that these three mutations rarely coincide [64], which suggests that they represent separate pathways to tumourigenesis [63].

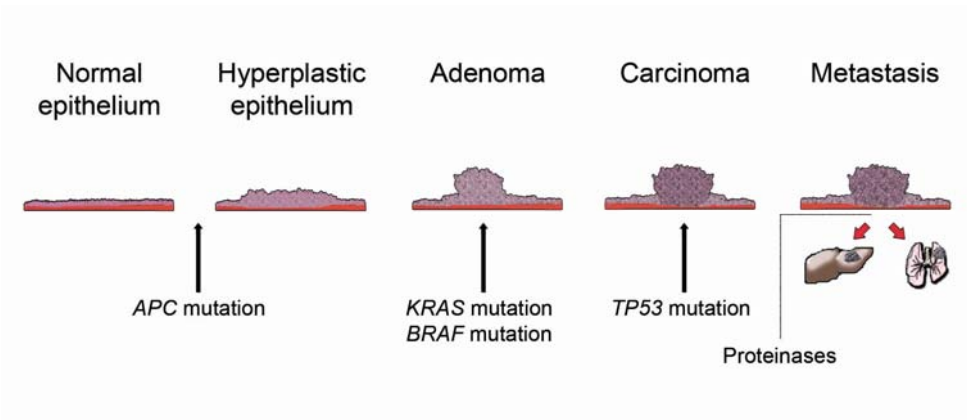


Figure 2. Genetic mutations in relation to tumour progression.

Genes encoding mismatch repair (MMR) proteins are also of importance in colorectal carcinogenesis. MMR proteins recognize and repair errors made by DNA polymerases, and *MMR* gene mutations can lead to both sporadic and familial cancers [8] and have been found to be highly linked to hereditary non-polyposis colorectal cancer (HNPCC) [review][65]. Microsatellite instability (MSI) refers to *MMR*-associated genetic alterations when the number of repetitive genomic nucleotides in a sequence is misplaced or altered, generating so-called micro satellites, in contrast to the wild type micro satellite stable (MSS) state [8]. *MMR*-mutations are believed to occur in approximately 15% of sporadic colorectal cancers [66]. Distinctions are also

made between MSI high (MSI-H) and MSI low (MSI-L) tumours, with 30% detected MSI defects as a suggested cutoff [67]. The majority of CRC have some MSI defects and fall into the MSI-L category. MSI-L and MSS tumours have been reported to have similar clinicopathological characteristics and prognosis, but, intriguingly, it has been shown that patients having MSI-H tumours have a better survival than patients with MSS and MSI-L tumours [68]. In metastatic CRC, however, MSI has been associated with an impaired survival [69], and MSI has also been reported to be a negative predictor of response to treatment with 5-FU [70]. Chromosomal instability (CIN) is another genetic pathway to human CRC that has been associated with poor prognosis [71], as well as with lack of response to treatment targeting the epidermal growth factor receptor (EGFR) [72].

## Clinical stage

The most important prognostic parameter in CRC is still the clinical stage of the disease [73]. In the last century, different classification systems have been introduced, of which the three most commonly used are the Dukes staging system [74], the Alster-Coller system [75] and the American Joint Committee on Cancer (AJCC) system, also known as the TNM system, which is the most commonly used classification today [76, 77].

TNM-staging consists of sub-terms, whereby T denotes tumour stage, N nodal stage and M metastatic stage, whereby M-stage is the most important prognostic factor [78]. Estimated survival according to the different stages summarised in Table 1. Although TNM-systems differ somewhat between different cancer forms, there is an attempt to conform the TNM-system [76, 79].

## T-stage

Tumour stage (T-stage) refers to the depth of tumour invasion into the wall of the intestine and overgrowth into nearby organs. T0, or Tis, indicates involvement of only the mucosa; T1 indicates growth through the muscularis mucosae and into the submucosa, T2 indicates invasion into, but not beyond, the muscularis propria, stage T3 tumours invade through the muscularis propria and into the subserosal layer of fat while stage T4 tumours penetrate the serosa/visceral layer of the peritoneum, or grow into nearby tissues or organs (Figure 3).

## N-stage

Nodal status (N-stage) indicates whether the cancer has spread to nearby lymph nodes and, if so, the extent of nodal involvement, whereby N0 = no cancer in nearby lymph nodes, N1 = cancer cells found in 1-3 regional lymph nodes and N2 = cancer cells found in 4 or more nearby lymph nodes. In 1991, the Working Party Report of the World Congress of Gastroenterology concluded that at least 12 lymph nodes should be dissected and examined by a pathologist after surgical resection of a CRC, for an adequate clinical staging [80] and fewer than 12 examines lymph nodes is considered a risk factor and may lead to administration of adjuvant chemotherapy.

## M-stage

Metastatic stage (M-stage) indicates whether the tumour has spread to distant organs, e.g. the peritoneum, liver, or lungs, and is determined as M0 = no distant spread or M1 = spread to distant organ(s) or distant lymph nodes.

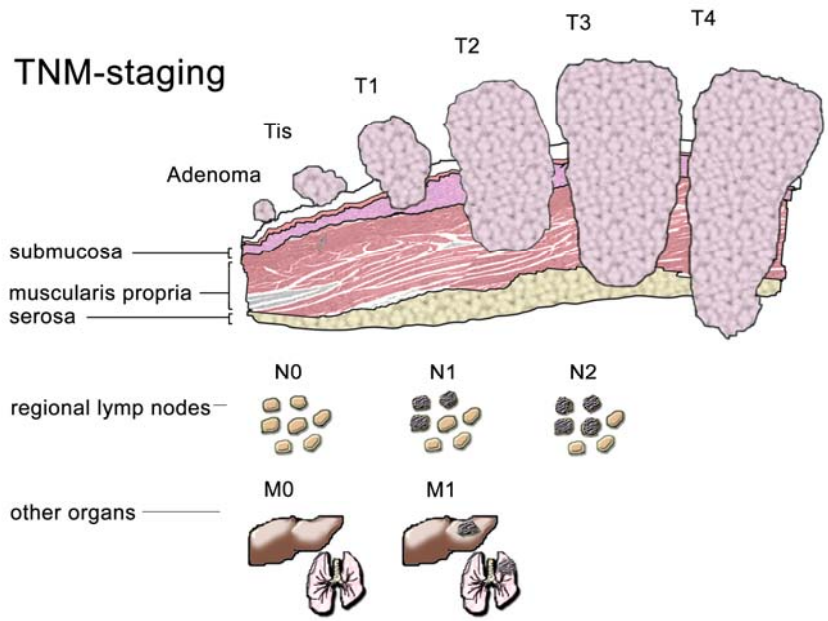


Figure 3. Schematics of TNM-staging (AJCC staging manual 6th ed.) [76].

# Impact of clinical stage on survival from colorectal cancer

While N-stage discriminates between stage II and stage III disease, positive M-status automatically places the patient into the stage IV category. The five-year survival of patients with Stage I disease is approximately 80-85%; 70-75%; for Stage II disease; 25-75% for Stage III disease; and only <3% for Stage IV disease [review][81](Table1). Adjuvant chemotherapy has so far only shown significant improvements in survival for patients having stage III disease [82], while surgery alone is believed to cure 80% of Stage II patients.

Table 1. Clinical stage according to 6th edition AJCC manual for staging of cancer [review][81]

| Stage | TNM | 5-year survival |
|---|---|---|
| 0 | Tis N0 M0 | >90 % |
| I | T1 N0 M0<br>T2 N0 M0 | 80-85 % |
| IIA<br>IIB | T3 N0 M0<br>T4 N0 M0 | 70-75 % |
| IIIA<br>IIIB<br>IIIC | T1-2 N1 M0<br>T3-4 N1 M0<br>T1-4 N2 M0 | 70-75 %<br>50-65 %<br>25-45 % |
| IV | T1-4 N1-2 M1 | <3 % |

# Other established prognostic factors

Another factor that is taken into account for prognostication of CRC patients is the differentiation grade of the tumour [73, 78], with the majority of CRC tumours being moderately differentiated [83]. Vascular invasion is also factor highly linked to prognosis, and denoted in the pathology report [78], as is perineural invasion [84]. Other factors considered in the clinical decision-making are age and gender. As previously mentioned, CRC is closely related to increasing age and approximately 90% of all CRC cases are diagnosed after 55 years of age [85]. Some studies have shown that younger patients tend to have more aggressive tumours [86, 87] but, on the other hand, younger patients are more likely to tolerate more aggressive chemotherapy and, in all, have a better survival [86]. Gender has also been found be of prognostic relevance with men having a worse prognosis [88], and while the lifetime risk of CRC is equal in both sexes, males seem to be more prone to receive

CRC at younger age [89]. It has also been shown that males have an increased post-operative mortality [90, 91].

Serum carcino-embryonic antigen (s-CEA) is at present the only marker used in clinical practice for monitoring of relapse in CRC patients [92]. European Group on Tumour Marker (EGTM) guidelines recommend that CEA should be measured for postoperative surveillance every 2-3 months for at least 3 years in stage II and III patients, and in patients who receive systemic treatment [93]. CEA levels have also been found to correlate with prognosis in CRC [94] and pancreatic cancer [95].

## Systemic treatment

In the past half century, a few standard chemotherapies have been used in both adjuvant and palliative settings for treatment of CRC. 5-fluorouracil (5-FU) was the first chemotherapeutic drug widely used for treatment of colorectal cancer and in the early 1990s, 5-FU was given combined with levamisole. Levamisole was however replaced with folinic acid later on, since the latter was found to be equally efficient and have fewer side effects [96, 97]. Another drug frequently used in the treatment of CRC is oxaliplatin, a platinum-based chemotherapeutic agent that was brought to use in the late 1990s. Oxaliplatin has been found to prolong progression free survival in patients with advanced CRC [98], however it has also been found to decrease the recurrence rate in patients with stage II CRC [99]. In the late 1990s another drug emerged; irinotekan, given in cases where 5-FU therapy was deemed ineffective. To date, 5-FU remains standard chemotherapy regimen for treatment of CRC and has, together with leucovorin and oxaliplatin, been shown to significantly improve disease free survival in curatively treated CRC patients having stage II or stage III disease [99-102].

In the early 2000s, novel therapeutic agents targeting the epidermal growth factor receptor (EGFR) emerged, e.g. cetuximab, currently used in the treatment of advanced colorectal cancer [103, 104]. *KRAS* mutations have in several studies been associated with primary resistance to EGFR-inhibition, since these mutations lead to constitutive activation of the RAS/RAF signalling pathway independent of EGFR activation by ligand binding [105-108].

## Neoadjuvant radiotherapy

Today, neoadjuvant radiotherapy (RT) is exclusively administered to rectal cancer patients, while colon cancer patients occasionally receive RT in the palliative setting. Short-term preoperative radiotherapy has been found to decrease local recurrence by 16% in rectal cancer patients, and improve survival by 21% [109]. Neoadjuvant RT has also been found to be more effective than adjuvant RT, even when adjuvant RT is administered in higher doses [110]. The desired effect of RT is to induce DNA damage, either directly or by generating free radicals, to such extent that the cells go into apoptosis [review][111]. In Sweden, preoperative RT is given either as short or long term regimen, where the short term RT regimen refers to a setting where the patient is treated with 5 Gy per day for 5 days followed by surgery within a week[review][112], and long-term RT to a setting is where the patient is treated with 1.8 Gy per day for 28 days, often in combination with 5-FU and leucovorin, followed by surgery after 4-6 weeks [review][112]. RT has previously been found to increase levels of MMP-2 and PAI-1 [113, 114].

## Tumour associated trypsin inhibitor

Proteinases consist of two major enzyme groups that break protein chains. Matrix serine proteinases (MSPs) are members of the serine proteinase inhibitor (serpin) family of proteinases, which break protein chains via hydrolysis [115] while matrix metallo proteinases (MMPs) are zinc dependent proteinases that interact with proteins using a zinc molecule [116, 117]. Both MSPs and MMPs are highly active in the degradation of the extracellular matrix (ECM) [118]. Trypsin, one of the earliest found proteases (1876) and member of the MSP family, breaks protein chains by hydrolysis of bonds between arginine and lysine [119] and is a 24kDa molecule, able of autocatalysis. Trypsin is secreted into the duodenum as an inactive zymogen form (trypsinogen-1/trypsinogen-2) which gets catalyzed into trypsin [120]. Trypsin-inhibiting enzymes counteract premature activation of trypsin in the pancreas and pancreatic ducts on the way to the site of function [121]. Trypsin has also been found to activate members of the MMP family [122], and premature activation of trypsin and proteases is believed to be the cause of the sometimes fatal condition acute pancreatitis [review][123], in which treatment with protease inhibitor has been found to decrease mortality in severe cases [124].

Tumour associated trypsin inhibitor (TATI; other names; pancreatic secretory trypsin inhibitor, PSTI, or serine proteinase Kazal type 1, SPINK1) is a 6kDa enzyme

comprising 56 amino acids [125], that was first discovered in the 1940s in pancreatic juice [126], where it was found to constitute 0.4 - 0.8% of the total protein mass [125] and is thought to inhibit 20% of the trypsin activity [review][127]. The acronym SPINK1 to the gene encoding TATI, which is located on chromosome 5 [128], mutation of which has been related to chronic pancreatitis [129, 130].

TATI is present in various tissue types in need of protection from proteolytic activities throughout the gastrointestinal tract, in particular in the pyloric antrum (~1240 µg/g), duodenum (~180µg/g) and colon (~160 µg/g)[131]. Some location dependent concentration differences of TATI have been found in normal colonic mucosa [Table 2][131].

The protective role of TATI in the colon has been demonstrated in studies where transgenic mice having high expression of TATI in the jejunal mucosa were found to be more resistant to drug induced injuries compared to wild type mice [132]. The main endocrine production of TATI is believed to occur in the pancreas, however, even after a total pancreatectomy, levels remain the same as prior to the procedure [133], and thus the enzyme must also be produced elsewhere. TATI is also present in lower concentration levels in the circulation under normal circumstances, where it most likely has the function to prevent activities of trypsin [134]. TATI has also been found to have strong-moderate inhibitory effects on other proteases including plasmin and urokinase, and weak inhibitory effects on tissue plasminogen activator, chymotrypsin, kallikrein and trombin [135].

Table 2. TATI concentration levels in normal colonic mucosa. Adapted from Freeman et al 1990 [131].

| **TATI in normal colorectal tissue** | **median µg/g** |
|---|---|
| Ascending colon | 230 |
| Transverse colon | 150 |
| Descending colon | 150 |
| Sigmoid colon | 210 |
| Rectum | 170 |

Elevated TATI concentrations in serum have been found in various non-malignant conditions, such as in patients having an impaired renal function [136], duodenal ulcers, bacterial peritonitis, urosepticemia, pneumonia [137], pancreatitis, and biliary

diseases [138-141], and the enzyme also acts as an acute phase reactant, rising after serious injury, surgery, and in response to inflammation [142-148].

## Role of TATI in cancer

Tumour invasion into the surrounding tissue requires degradation of the basal membrane and the extracellular matrix (ECM), a process for which proteolytic activities are required [review][149]. Interactions of the tumour cells with normal tissue is believed to occur in three steps; attachment, dissolution of the ECM and migration, whereby proteinases play a crucial role in the second step [review][149]. TATI was for the first time associated with cancer in 1982, when it was found in the urine of an ovarian cancer patient [150]. In the early research related to the role of TATI and trypsin in tumour progression, researchers believed that TATI merely reflected the activities of trypsin, with trypsin being the actual "bad-guy" [151, 152]. Over time, however, TATI has been found to play a more extensive role than being merely a trypsin inhibitor, not least in a functional context. As summarised in Table 3, the prognostic role of TATI has been investigated in several different cancer forms, with somewhat diverging results [153-163]

Although s-TATI is not suitable for diagnostic purposes due to low sensitivity [review][164], it has been suggested as a complementary biomarker for monitoring patients with gastric ulcers in risk of developing malignancy [147, 165], and as a diagnostic marker for ovarian cancer [166].

Elevated levels of TATI in serum in various malignant conditions have been proposed to be caused by leakage from the tumour [148] or an immunological tumour response [151]. TATI has been found to correlate to the levels of trypsinogen-2 in cyst fluids of ovarian cancer patients [167] as well as in colon cancer cell lines expressing trypsinogen-2 [168]. In bladder cancer, TATI expression has been found to decrease in tumours of higher stage and grade [169], where disruption of the balance between t-TATI and trypsin was suggested as a mechanism for tumour progression [169].

In 2008, Gouyer et al found TATI to be an autocrine transforming factor, potentially involved in both early and late colon cancer progression [170]. TATI was demonstrated to mediate an invasive behaviour in human adenoma and carcinoma cells of the colon and breast through phosphoinositide-3-kinase, protein kinase C and Rho-GTPases/Rho kinase-dependent pathways Additionally, transfecting cells with mutant TATI, thus eliminating TATI wild type function, downregulated tumour growth, angiogenesis and the expression of several metastasis-related genes [170].

Table 3. Summary of results from previous studies on TATI and prognosis in various cancer forms.

| Author, year, (ref) | Carcinoma type | n | Location and cut-off, (median range / µg/L) | Important findings relating to prognosis |
|---|---|---|---|---|
| Gion et al., 1991 [153] | Oesophagal | 71 | Serum (RIA) 20.1 µg/L (20.6) | S-TATI is associated with decreased overall survival. |
| Venesmaa et al., 1994 [154] | Ovarian | 66 | Serum (RIA) 22 µg/L (87.3) | S-TATI is associated with poor overall survival, remaining significant in multivariable analysis including age, stage, histological grade, primary residual tumour and CA 125. |
| Venesmaa et al., 1998 [155] | Ovarian (Stage III) | 98 | Serum (RIA) 22 µg/L (n/a) | S-TATI is associated with poor prognosis in patiens having stage III disease with ≤2cm residual tumour, remaining significant in multivariable analysis adjusted for age, residual tumour size, histologic grade and histologic type. |
| Vartiainen et al., 2001 [156] | Ovarian | 146 | Serum (RIA) 21 µg/L (n/a) | S-TATI is associated with poor prognosis in stages III & IV, but not in multivariable analysis. |
| Paju et al., 2001 [157] | Renal cell | 188 | Serum (RIA) 16 µg/L (15) | S-TATI is associated with poor prognosis, also after adjustment for stage and age. |
| Kelloniemi et al., 2003 [158] | Bladder | 157 | Serum (RIA) 21 µg/L (n/a) | Highest s-TATI quartile is associated with poor prognosis, also after adjustment for age, stage, urine cytology and urine TATI. |
| Paju et al., 2004 [159] | Ovarian | 119 | Tumour tissue (IHC) Serum (RIA) 22 µg/L (serous;15/mucinous;25) | S-TATI is not found to be of prognostic importance. |
| Wiksten et al., 2005 [160] | Gastric | 336 | Tissue (IHC) (>50% cells) | T-TATI is associated with a good prog¬nosis, however not remaining significant in multivariable analysis, including TNM stage, age, gender, tumour location, Lauréns classification, Borrman type. |
| Y.-C. Lee et al., 2007 [161] | Hepatocellular | 258 | mRNA tissue (RT-PCR) (TATI to control gene ratio >1) | Overexpression of TATI mRNA issignificantly associated with an impaired 5year-survival in all stages of HCC. |
| Leinonen et al., 2010 [162] | Prostate (hormonally treated) | 186 | Tissue (IHC) (>10% cells with highest intensity) | TATI (SPINK1) is significantly associated with shorter time to clinical/biochemical recurrence, also in multivariable analysis adjusted for Gleason score, lymph node status, surgical margin status, age and preoperative PSA. |

# TATI and EGFR

Extracellular stimuli, e.g. growth factors, cytokines, mitogens and hormones, interact with transmembrane receptors such as tyrosine kinase receptors, as a form of biological communication [8]. Phosphorylation of proteins and its importance in signal mediation has been known since the 1950ies [171]. The epidermal growth factor receptor (EGFR) is a 170 kDa transmembrane tyrosine kinase receptor protein which upon activation by binding of a ligand functions as a mediator of the extracellular signal into the cell, thereby activating intracellular transcription factors and proliferative mechanisms [172](Figure 4). TATI has been found to have a similar molecular structure to EGF, with approximately 50% sequence homology, and a similar size [173-175].

Early experiments revealed that endothelial cells, mouse fibroblasts [176-178], and rat pancreatic cancer cells [179] treated with TATI were more proliferative. In studies on human cancer cells, TATI treatment was found to promote cellular motility of HT-29 colon cancer cells [180], and Colony-29 adenocarcinoma cells [132]. Intriguingly, further experiments on the pro-migratory effect of TATI in a primary murine cell line revealed that this was only seen in EGFR positive cells, in contrast to an identical EGFR negative cell line, that remained unaffected by treatment with both TATI and EGF [132]. Moreover, the pro-migratory effect of TATI on EGFR positive Colony-29 cells was successfully inhibited by anti-EGFR treatment [132]. TATI has been found to induce phosphorylation of EGFR in Colony-29 cells, in a gradual, prolonged fashion, ranging up to 30 minutes, in contrast to short intense phosphorylation by EGF, which diminished after 15 minutes [132]. In that study, TATI had no effect on proliferation [132], but in a later study, TATI was demonstrated to bind to EGFR and induce proliferation in pancreatic cancer cells [181]. Moreover, this proliferative effect was demonstrated to occur via activation the mitogen activated protein kinase (MAPK) pathway [181]. Activation of JAK/STAT and PI3K pathways was also observed, but to a minor extent [181]. Another recent study on prostate cancer cells showed results in line with the previous studies, where TATI was found to induce EGFR phosphorylation, which was sustained for 90 minutes but with lower intensity, in comparison with EGF, binding of which induced a sustained intense phosphorylation for 10 minutes [182]. Furthermore, TATI had substantial effects on both proliferation and invasion, and knock-down of SPINK1 rendered the cells less proliferative and motile [182]. It is also noteworthy that the effect of TATI could not be completely inhibited by anti-EGFR treatment [182].

STAT3 (signal transducer and activator of transcription 3) is one of seven members of the STAT family of cytoplasmic latent transcription factors [183, 184]. STAT3 is activated by tyrosine phosphorylation in response to stimuli of several receptors including EGFR [review][185, 186] and it has been found to play an important role in several cancer forms [review][185], including colorectal cancer, where its expression has been associated with an impaired survival [186, 187]. Phosphorylated STAT3 has been found to mediate tumourgenic properties in cell lines and mouse models [188] and, moreover, STAT3 activation has been found to regulate MMP-2, and thereby increase tumour aggressiveness in melanoma cells [189]

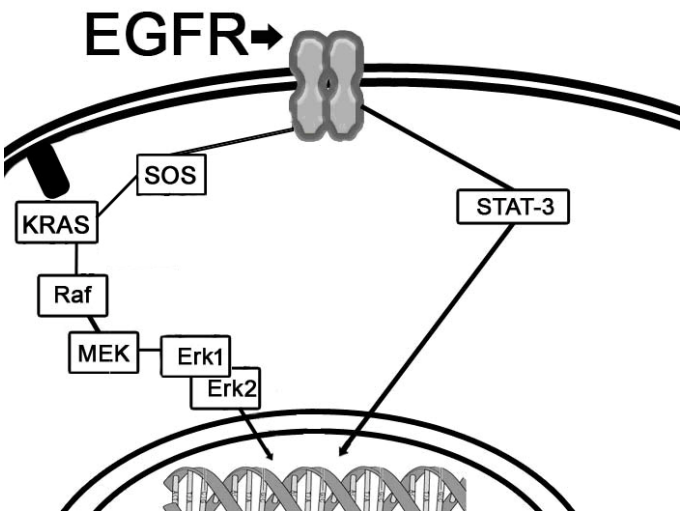

Figure 4. Examples of key proteins mediating EGFR signalling.

Extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) are members of the mitogen-activated protein kinase super family that translocate into the nucleus upon activation, and play a crucial role in cell cycle progression from G1 phase to S phase [190]. It has been found that ERK1/2 are activated by stimulation of a variety of receptors, including EGFR [191]. Nuclear pERK1/2 expression has previously been associated with better survival in tamoxifen untreated breast cancer patients [192], but with poor prognosis in CRC and hepatocellular carcinoma [193, 194]. In hepatocellular carcinoma, pERK1/2 expression has also been associated with *KRAS*-mutation [194]. Wild type KRAS protein is bound to guanosine diphosphate (GDP)in its inactive form [33]. Among the most well-characterized effectors of Ras are members of the Raf family, that regulate the MAPK-pathway [review][195]. Growth factors activate guanine nucleotide exchange factors (GEF) which stimulate the formation of guanosine triphosphate (GTP) which in turn activates KRAS [196]. Active KRAS controls signalling pathways that determine Ras-induced cellular

responses such as cell proliferation, survival, differentiation and motility [197]. Ras guanosine triphosphatase (GTPase) promotes hydrolysis of GTP into GDP thereby inactivating KRAS [197]. Mutated *KRAS* on codon 12,13 and 61, retains a GTP bound active state, due to resilience of inactivation by GTPase [197].

# Methods

## Patient cohorts

### Consecutive cohort from Västerås (Papers I, II and IV)

The major results in this thesis derive from a consecutive cohort of 337 CRC patients, 167 (49.6%) women, and 170 (50.4%) men surgically treated in the hospital of Västerås, between 2000 and 2003. Fresh tumour tissue was sampled from 249 (73.9%) patients at surgery and snap-frozen. Paraffin embedded tumour tissue was available from a total of 320 (95.0%) of the surgical specimens and serum samples were obtained from 325 (96.4%) patients prior to surgery. Benign-appearing colorectal mucosa (n = 105) and lymph node metastases (n = 67) had also been sampled from a subset of cases. Two hundred and eighty three patients were curatively treated. The distribution of disease stages is shown in Figure 5.

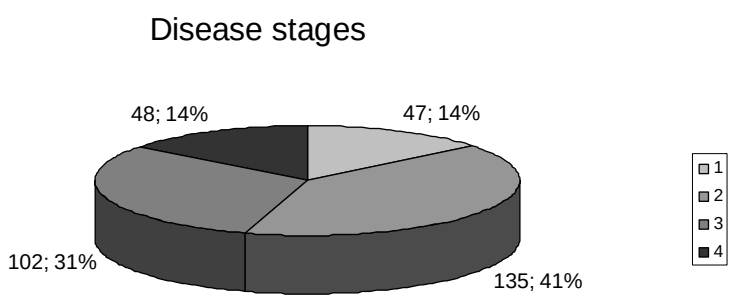


Figure 5. Distribution of disease stage in Västeråscohort-patients.

### Test-cohort from Malmö (Paper I)

In paper I, an additional cohort of 118 CRC patients, 61 (52%) women and 57 (48%) men, surgically treated in Malmö University Hospital between January 1999 and March 2002, was used as a test cohort. The cohort was designed to comprise a fairly equal distribution of CRC patients with stage I-III disease

### Rectal cancer cohort (Paper III)

In paper III we used a small prospective patient cohort consisting of 77 patients, diagnosed and treated for rectal cancer at Skåne University Hospital in Malmö, between 2003 and 2007. Twenty-four patients were excluded, due to reclassification of tumours into high-grade dysplasia (n = 8), synchronous tumours (n = 3), declination to participate in the study (n = 4) or for logistic reasons (n = 1). Thus, a remaining number of 53 patients, 36 (67.9%) males and 17 females (32.1%), were eligible, among whom one group was treated with short-term neoadjuvant RT (n = 20), another group with long-term neoadjuvant RT (n = 21), and a control group was treated with surgery alone (n = 12). No patients in this cohort received neoadjuvant chemotherapy. Biopsy samples from normal rectal mucosa, cancer, and serum were collected at baseline for all patients. For long-term RT treated patients, biopsies and serum samples were collected halfway into treatment and at the end of each RT treatment course. Tissue and serum was obtained from all patients at surgery, whereby serum was sampled before surgical incision to exclude acute-phase reactant response, and four weeks after surgery. RT treatment was prescribed and administered using a conventional three-field technique, whereby two 10 MV fields are administered bilaterally and one 6 MV field is administered posteriorly, directed towards the tumour, rectum and collateral regions. The short-term regimen RT was administered in a 5 day interval, 5 Gy per fraction adding up to a total of 25 Gy with surgery performed 3 days after completed RT. For the long-term regimen treatment, patients received 10 Gy per week, 2 Gy per day, during a 5 week period adding up to 50 Gy. This patient group then had a recovery period of approximately 5 weeks before surgery.

## Tissue micro array technique

The tissue micro array (TMA) technique is a high-throughput approach for analysis of several minute tumour-samples in one session, thereby decreasing the amount of

antibody and tissue material required for evaluation, in contrast to the use of full-face tissue sections. The technique was developed in 1998 by Kononen et al. [198] and is an extension of the multitumour or "sausage" block invented by Battifora [199]. Using a TMA constructing device, manual, semi-automated or fully automated, cores of tissue measuring 0.6 – 2.0 mm diameter are taken from the desired donor block(s), and mounted into a recipient block.

Figure 6. Transfer of tumour cores from a donor block into a recipient block.

The TMA-technique is well-established for high throughput analysis [200] and, while the technique might not be optimal for some heterogeneously expressed markers, this issue usually does not constitute a major problem [201, 202] .

Figure 7. Sectioning of a TMA block using a microtome.

# Immunohistochemistry and antibodies

All studies in this thesis are based on results from immunohistochemical (IHC) stainings. IHC is an antibody-based technique for detection of proteins in tissue. In brief, 4µm full-face or TMA sections are deparaffinised with Xylene, rehydrated using decreasing alcohol concentrations and thereafter heated. The removal of paraffin allows for dipolar fluids to get into direct contact with the tissue, while the rehydration renders the cells permeable. Formalin-fixed paraffin embedded tissue needs to be pre-treated before IHC staining due to formation of methylene bridges between proteins [203]. Pre-treatment consists of a heating process, also called heat induced epitope retrieval (HIER) or antigen retrieval that induces energy to protein bindings and makes binding sites susceptible to antibody binding. It is also proposed that the treatment disrupts formalin-induced intra- and intermolecular cross links of proteins [204]. The antibody for detection of TATI (6E8) used in this thesis was developed and validated at Helsinki University Central Hospital [205]. This antibody was used in all IHC stainings, as well as in the analysis of TATI in serum, using a time resolved immunofluorometric assay. Different commercial antibodies were used for detection of CEA, EGFR, ERK1/2, and STAT3 (Table 3).

Table 3. Summary of antibodies used in papers I-IV.

| Marker | Manufacturer | Clone | Dilution | Processing system | Paper |
|---|---|---|---|---|---|
| TATI | Helsinkki Uni. | 6E8 | 1:500 | DAKO Techmate 500/IFMA | I-IV |
| TATI | Helsinkki Uni. | 11B3 | | IFMA | II,IV |
| CEA | IBL Kit (RE59101) | - | - | IFMA | I-III |
| EGFR | Zymed Labs. | 31G7 | 1:25 | DAKO Techmate 500+ | IV |
| pERK1/2 | Cell Signaling | - | 1:100 | DAKO Techmate 500+ | IV |
| pSTAT3 | Cell Signaling | D3A7 | 1:100 | DAKO Techmate 500+ | IV |

For all stainings, the DAKO Autostainer (DAKO; Glostrup, Denmark) was used according to the manufacturer's instructions. All IHC stainings were manually annotated, usually by 2 or 3 associates. Both the fraction of positive cells and intensity of staining were annotated separately for each core. Apart from Paper I, where only the fraction of positive cells was taken into account, a multiplier of fraction and intensity was constructed in order to get a continuous score, a system used previously in tissue based research [206] as well as in IHC protocol design [207].

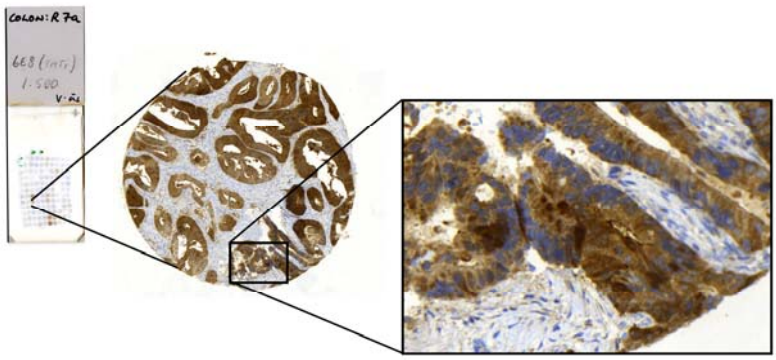


Figure 8. Immunohistochemical staining of colorectal cancer with TATI antibody 6E8, diluted 1:500.

# Statistics

Since TATI concentration/expression is skewely distributed, mainly non-parametric analyses were used throughout this thesis. Mann-Whitney U-test was used for assessment of differences in biomarker levels according to various patient- and tumour characteristics. Chi-square test and Spearman's correlation analysis were used to examine the associations between the investigative biomarkers and clinicopathological parameters. Kaplan-Meier curves and the log rank test were applied to illustrate differences in survival in strata according to the expression and concentrations of the investigative biomarkers [208]. Cox regression analysis was applied to examine the prognostic relevance in univariable and multivariable settings, calculating hazard ratios (HRs) with a 95% confidence interval, which means that there is a 5% chance of type I error i.e. the null hypothesis being true. The endpoints used throughout the studies in this thesis, are in accordance with Punt et al [209], whereby overall survival (OS) is defined as time to death, irrespective of cause,, disease free survival (DFS) is defined as time to any event, irrespective of cause, including all cancer-related events, and time to recurrence (TTR) defined as any event, including all recurrences and death, related to strictly the same cancer. Optimal prognostic cut-offs were calculated by receiver operating characteristics (ROC) curve analysis and classification and regression tree (CRT) analysis, in papers II-IV. Analyses were carried out using

mainly SPSS 16.0.1 (Chicago, USA), but also Medcalc 9.6.2 (Mariakerke, Belgium) and STATS direct 2.7.2 (Altrincham, United Kingdom).

## Solid-phase time-resolved immunofluorometric assay

Serum concentrations of TATI as well as CEA were analysed using a modified version of solid-phase time-resolved immunofluorometric assay (IFMA) [205]. This sandwich assay type method came to use for analysis of TATI in 1993 and has a higher range of detection than the previously used radio immuno assay (RIA), which has a detection limit of 1-50 µg/L [150], while Streptavidin/biotin IFMA (SAB-IFMA) has a lower detection limit of 0.05 µg/L.

A modification to the method is the use of 2 monoclonal antibodies, TATI (6E8) as solid phase antibody bound to the wells of a polysterene microtitration plate and a Europium3+-labelled TATI mAb (11B3) used as a catcher, thereafter analysed by a fluorometric reader. In a first step, wells are coated with Streptavidin solution (S4762, Sigma Chemical Co., St Louis, MO) and incubated overnight. Then the wells are treated with glutaraldehyde for 30 min and later with saturation solution containing bovine serum albumin for blocking of unspecific binding, and washed with Tris-HCI buffer. Thereafter biotinylated mAb TATI antibody (6E8) in assay buffer is added to the wells, incubated for 1.5 hours at room temperature, and then the plate is rinsed with washing solution. Patient serum, 25 µL, is then added to each well, including a control well, and incubated for 1 hour. After washing, each well is treated with Europium-labelled catcher TATI mAb (11B3) in assay buffer for 1 hour. The unbound fraction is removed by washing, enhancing solution added, and the bound Eu3+ measured by time resolved fluorometer (Wallac Victor2 1420, Perkin Elmer Life and Analytical Sciences, Turku, Finland).

## Pyrosequencing analysis

For paper IV, we analysed *KRAS* mutation status using pyrosequencing [210], a method for determining the order of nucleotides in a gene segment. Amplification of DNA from codon 12 and 13 of the *KRAS* gene were performed for each patient by use of PCR, and the resulting DNA-product was analysed for mutation in the pyrosequencing assay. In this process, a sequencing primer is hybridized to a single-stranded PRC-amplicon serving as a template, and incubated with the enzymes DNA-polymerase, ATP sulfurylase, luciferase, apyrase as well as with the substrates

adenosine 5' phosphosulfate (APS), and luciferin. In short, deoxyribonucleotides are sequentially added to the DNA-template in the order represented by the wild-type gene. If the added nucleotide is complementary to the single stranded DNA, it binds to the DNA and pyrophosphate (PPi) is released proportionally to the amount of bound nucleotide. ATP sulfurylase converts PPi to ATP in the presence of APS and ATP mediates transformation of luciferin to oxyluciferin. This transformation generates a visible light detected as a peak in the data output. The height of each peak correlates to the number of nucleotides incorporated. Apyrase, a nucleotide-degrading enzyme, degrades unbound nucleotides and ATP and following this degradation, another nucleotide is added. The result of the sequencing is summarised and analysed in a pyrogram which provides fully quantitative allele data.

# The present investigation

Prior to this thesis work, the prognostic role of TATI had been reported in several cancer forms, but not CRC. The overall aim of the present investigation was therefore to examine the potential relevance of TATI as a prognostic biomarker in CRC, both as assessed in tumour tissue and serum.

## Paper I

### Aims

In paper I we investigated the association of immunohistochemical TATI expression in tumour tissue (t-TATI) with clinicopathological parameters and survival from CRC. For this purpose, we examined two independent patient cohorts; whereby the Malmö cohort (Cohort I, n =118) was used as a test cohort and the cohort from Västerås (Cohort II, n = 320) as a validation cohort. The percentage of positive tumour cells was estimated and assigned values of 0, 5 or multiples of 10%. The intensity of the expression was assigned a value of 0, 1, 2 or 3.

### Summary of results

In this paper, we show, for the first time, that TATI expression is an independent prognostic marker in CRC. In cohort I, where TATI could be evaluated in 114/118 (96.6%) cases, an optimal cutoff at 50% positive cells was established for prognostication, whereby TATI was found to be associated with a shorter OS (HR = 2.42; 95% CI = 1.38-4.26), remaining significant in multivariable analysis including age, gender, disease stage, differentiation grade and vascular invasion (HR = 1.80; 95% CI = 0.99-3.27, P = 0.05). Further on we found an increased risk of metachronous liver metastasis (HR = 3.97, 95% CI = 0.99-15.88, P = 0.05), but this did not remain significant in multivariable analysis (HR = 3.69, 95% CI = 0.87–15.67, P = 0.08).

Proceeding with cohort II, TATI could be successfully evaluated in 310/320 (96.9%) tumours. Using the cut-off established from the analysis of Cohort I, high TATI expression was found to be significantly associated with an impaired OS in all patients (HR = 1.73, 95% CI = 1.14-2.64), and a significantly reduced DFS in curatively treated patients (HR = 1.52, 95% CI = 1.03-2.25). In multivariable analysis including adjustment for age, gender, disease stage, differentiation grade, vascular invasion and CEA concentration levels, t-TATI remained significantly associated with a shorter OS (HR = 1.82, 95% CI = 1.19-2.79) and shorter DFS (HR = 1.56, 95% CI 1.05-2.32). While t-TATI had no significant impact on TTR, high t-TATI expression was significantly associated with a reduced time to liver recurrence, and this remained significant in multivariable analysis (HR = 2.85, 95% CI 1.43-5.66).

Correlation analyses between t-TATI and conventional clinicopathological factors revealed a positive association between t-TATI expression and higher age (>75 years) in Cohort I (P = 0.008), but not in Cohort II, although there was a trend towards older patients having tumours with higher t-TATI expression. Otherwise, t-TATI was not associated with any clinicopathological parameters using the dichotomized variable of high and low t-TATI expression and chi-square test. However, analysis of the full distribution of the best staining score in relation to T-stage, N-stage and clinical stage revealed that t-TATI was significantly lower in tumours of more advanced T-stage (P = 0.031), with similar, however non-significant, trends for N-stage and clinical stage. There was also a non-significant trend towards lower t-TATI expression in cancers of the left colon compared to the right colon.

## Discussion

The results from Paper I demonstrate, for the first time, that high tumour-specific expression of TATI is a factor of poor prognosis in CRC and, moreover, seems to promote a tumour phenotype with predilection of liver metastasis. A strength of the study is the use of two independent patient cohorts. In order to set an optimal prognostic cutoff, we first analysed TATI expression in a smaller, retrospectively collected cohort, used as a test set. A dichotomised variable with the best prognostic separation was constructed, taking only the fraction of positively staining cells into account, since the intensity of staining had no prognostic value. The association of high t-TATI expression and poor prognosis was then validated in the second cohort with prospectively collected tumour samples, using the same cutoff at 50% positively staining cells. Since these tumours have been identified within a prospective cohort, the risk of sampling bias should be reduced. In addition, more extensive information on clinicopathological characteristics, treatment and follow-up was available for the patients in Cohort II. In 2010, an extended follow-up was performed for Cohort II,

whereby the impact of TATI on survival was slightly decreased, in particular related to OS, where it changed to borderline significance. Otherwise, the revision of survival data did not alter the results in any major way, and the prognostic significance of t-TATI was similar to the visually established cutoff when more objective methods, i.e. ROC and CRT analysis, were applied for establishment of the best cutoff. Moreover, using a multiplier of fraction and intensity of staining did not significantly alter the findings related to the prognostic value of t-TATI.

It has previously been suggested that loss of TATI expression in tumour tissue could occur due to hydrolysis by mucosal enzymes prior to extraction [131]. Handling of tissue in routine pathology requires a fast and standardised processing of the tissue. Formalin fixation is a time-dependent process and optimal formalin fixation time for IHC is generally 24 hours and if the start of formalin fixation is delayed by 12h in (4˚C), IHC staining can potentially get 50% weaker [207]. However formalin fixation treatment has by itself a degenerative effect on tissue samples and formalin over-fixation or prolonged formalin fixation may in some cases cause an impaired immunoreactivity. For instance, formalin fixation has been shown to cause decreased IHC staining of Ki-67 in HT29 cell xenografts from 35% to 11% if fixation was prolonged by 24 h [211]. An impaired immunoreactivity will most likely appear as a decreased staining intensity, while the fraction of the stained cells often remains the same, speculatively because it reflects differences in the histoprocessing of the surgically obtained tissue samples [212]. Biomarker studies on t-TATI expression have previously also used the fraction of positive cells rather than the staining intensity [162], [207]. Since the results from this paper are based on analyses of two entirely different cohorts, the fixation issue should be a minor problem.

The finding of a lower t-TATI expression in more advanced TNM stages is line with a previous study on bladder cancer [169]. In that study, concentrations of trypsinogen-1 and trypsinogen-2 remained largely unchanged in higher clinical stages, leading the authors to suggest that disruption of the balance between TATI and trypsinogen in the tumours could lead to tumour progression [169]. Notably, while the association of lower t-TATI expression with more advanced N-stage did not reach significance, the proportion of lymph node samples expressing high levels of TATI was only 12/67 cases (18%) compared to the primary tumours, where 151/310 cases (49%) expressed TATI. The full distribution of TATI best score in benign-appearing colorectal mucosa, primary tumour and lymph node metastases is shown in Figure 9A-C. TATI expression in lymph node metastases was significantly lower (Wilcoxon $Z = -5.201$, $P < 0.001$), than in the primary tumours (Figure 9D), indicating that, although t-TATI expression in the primary tumour is a factor of poor prognosis, it may be downregulated once metastatic disease has been established. It would

therefore be of interest to compare the expression of TATI and trypsin in primary colorectal cancer and metastatic deposits in future studies.

While no association was seen between t-TATI expression and time to recurrence, high t-TATI was significantly associated with a reduced time to liver metastasis. Future studies are warranted to elucidate the mechanistic basis for these findings, but it is noteworthy that experimental targeted therapy with a synthetic MMP inhibitor, batimastat, has been shown to increase the number of liver micro metastases from breast and lymphoma cells in mice [213].

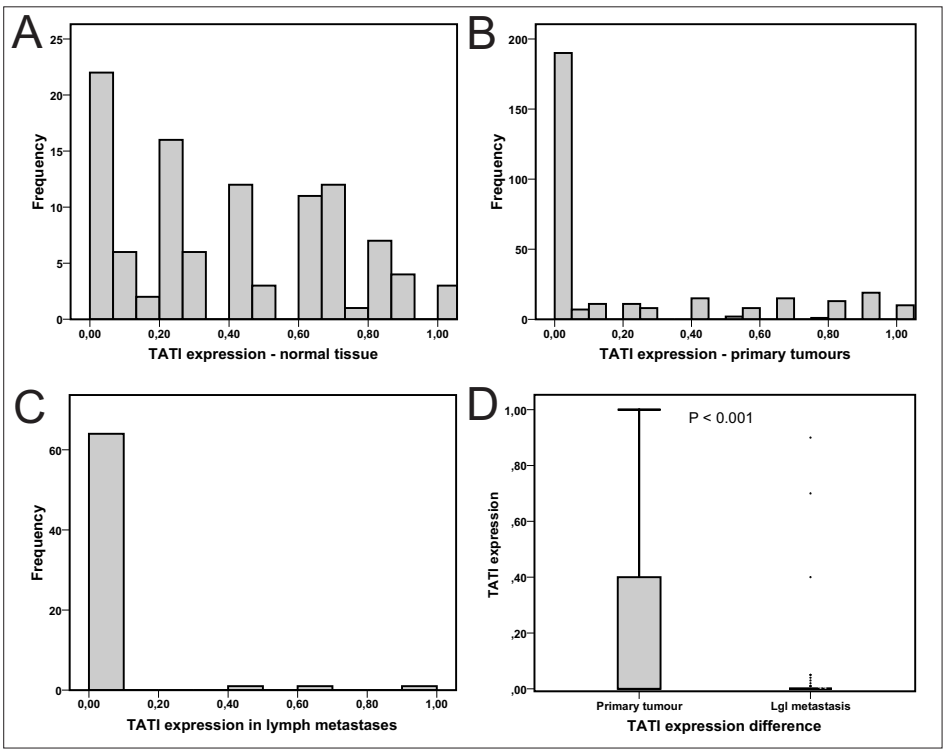


Figure 9. T-TATI distribution in (A) benign mucosa, (B) primary tumours (C) lymph node metastases, (D) T-TATI distribution difference between primary tumours and lymph node metastases.

# Paper II

## Aims

In Paper II, we furthered our aim to investigate the prognostic value of TATI concentrations in serum (s-TATI) of CRC patients. In addition, we wanted to investigate the intercorrelation of s-TATI with t-TATI and established clinicopathological factors including s-CEA. For this purpose, serum samples from 325 patients in Cohort II from Paper I were analysed using an immunofluorometric assay. S-TATI concentrations were divided into in quartiles as well as a dichotomized variable of low vs high expression based on ROC curve analysis.

## Summary of results

S-TATI was successfully analysed in serum samples from 324/325 (99.7%) patients, with concentrations ranging from 4.4 µg/L to 169 µg/L (mean 23.5 µg/L, median 13.5 µg/L) (Figure 10), which is in line with earlier studies on s-TATI in CRC patients [146]. Significantly higher s-TATI levels were found in patients with distant metastases ($P = 0.020$), and s-TATI was also higher in patients with more advanced T- an N-stage, although these associations did not reach statistical significance. S-TATI concentrations were associated with higher age at diagnosis ($P < 0.001$), similar to the findings from Cohort I in Paper I. Moreover, patients with rectal cancer had significantly higher s-TATI levels compared to patients with colon cancer ($P = 0.001$). Preoperative RT had been given to 84/107 patients with rectal cancer, and in the full cohort, a total number of 85 patients had received neoadjuvant RT, among whom s-TATI levels were lower than in patients who had not received neoadjuvant RT ($P = 0.001$). S-TATI concentrations were higher in patients having tumours located in the right colon compared to the left colon ($P = 0.056$, borderline significance). Notably, no correlation was found between s-TATI concentrations and t-TATI expression levels, and there was no correlation between s-TATI and s-CEA.

Survival analyses were performed in all patients, curatively treated patients as well as in groups of stage II and stage III patients, whereby s-TATI was found to be a strong, independent predictor of a reduced OS in all subcategories, adjusted for age at surgery, gender, disease stage, differentiation grade, lymphatic or vascular invasion. s-CEA was also a marker of poor prognosis, but this was less evident in subgroup analysis of patients with stage II and III disease.

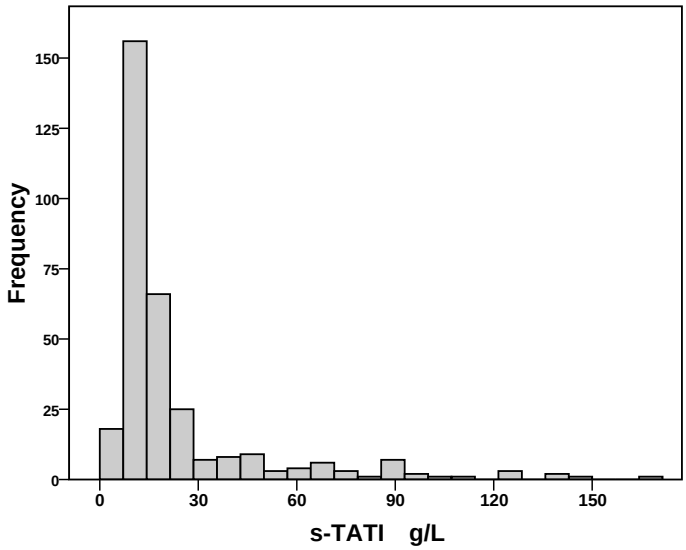


Figure 10. Distribution of s-TATI concentrations.

## Discussion

The results from Paper II show that TATI concentrations in serum are a strong factor of poor prognosis in CRC, even after adjustment for age, gender, disease stage, differentiation grade, lymphatic or vascular invasion, s-CEA, and t-TATI. However, although s-TATI was a stronger indicator of poor prognosis than t-TATI, it was not associated with time to liver metastasis. The lack of an association between s-TATI concentrations and t-TATI expression was somewhat unexpected, but a previous study on renal cancer also failed to show such an association [214]. Altogether, these findings suggest different mechanisms underlying elevated levels of TATI in serum and tumour tissue, and that elevated s-TATI levels are not due to leakage from tumours into the systemic circulation.

As s-TATI has previously been associated with an impaired renal function [136], this might represent a potential confounder in the survival analyses. A limitation to this study is therefore the lack of information regarding s-creatinine levels. However, as renal function usually diminishes with age, and the independent prognostic value of s-TATI was not altered by inclusion of age in the multivariable analyses, renal function should not be a major confounder.

This study is, to our knowledge, the hitherto largest study on the prognostic value of s-TATI in CRC. Previous studies have mainly focused on its diagnostic value [165, 215], but in a study comprising several cancer forms, high s-TATI were found to correlate with liver metastasis in CRC (n = 55) [146]. The significant difference in s-TATI concentrations between the colon and rectum, being lower in the latter, is noteworthy, although the prognostic impact did not differ according to tumour location Additional studies are needed to clarify whether this finding is coincidental or actually mirrors different tumour biological properties of colon and rectal cancers. As all rectal tumour samples were taken from post treatment surgical specimens, it could also be speculated that TATI levels are modified by neoadjuvant RT. Indeed, s-TATI levels were significantly lower in tumours treated with neoadjuvant RT compared to untreated tumours. As denoted in Paper I, there was a trend towards higher t-TATI levels in right-sided compared to left-sided colon cancers, but this was not significant and there was no significant difference in t-TATI expression between tumours located in the colon and rectum (data not shown).In contrast to s-TATI, s-CEA concentrations did not differ according to tumour location. Given the higher frequency of MSI tumours in the right colon, which are often associated with a Crohn's-like lymphocytic infiltrate [216, 217], it would be of interest to investigate the association of t-TATI, s-TATI and MSI status in CRC in future studies.

In this study, s-TATI was analysed using a modified time-resolved enzyme-linked assay [205, 214], a method similar to ELISA. In comparison with radioimmunoassay (RIA), the most frequently used method for assessment of TATI concentrations in serum in earlier studies, time-resolved enzyme-linked assay has a 3000-fold analytical range [205].

# Paper III

## Aims

To follow up on the findings in paper II, where we found significantly lower s-TATI concentrations in rectal cancer patients and in patients treated with neoadjuvant RT, the aim of paper III was to investigate the effects of RT treatment on levels of TATI in tumour tissue and serum. For this purpose, we analysed TATI concentrations in normal rectal mucosa, tumour tissue and serum, sampled at different timepoints during treatment, from 53 rectal cancer patients included in a randomized trial. The patients had either received short-term RT (n = 20, 37.7%), long-term RT (n = 21, 39.6%) or no RT (n = 12, 22.6%). Further aims of this paper were to analyse the

association between s-TATI and s-creatinine concentrations and to validate the prognostic value of s-TATI and t-TATI.

## Summary of results

S-TATI concentrations ranged from 4.28-62.49 µg/L (median 9.06 µg/L). There were no significant differences in neither s-TATI concentration nor t-TATI expression before, after or during RT in patients who received RT. In patients treated with short-term RT, s-TATI concentrations were significantly higher in samples taken post surgery compared to serum drawn prior to RT or prior to surgery (Wilcoxon $Z = -3.366$, $P < 0.001$). S-TATI was significantly associated with a reduced OS in samples drawn at all timepoints, while t-TATI was significantly associated to an impaired OS only as assessed in tissue from the specimens obtained at surgery, not in pre-surgical biopsies ($P = 0.045$).

T-TATI was found to correlate with male gender (Spearman's correlation = 0.406, $P = 0.008$). S-TATI correlated with higher age ($P = 0.001$) and increased s-creatinine concentrations in serum drawn prior to surgery ($P = 0.041$). s-TATI at follow-up was not associated with s-creatinine levels and t-TATI was not associated with s-creatinine levels at any timepoint. There was no significant association between s-creatinine levels and age. In line with previous findings from Paper II, there was no significant association between s-TATI and t-TATI concentrations.

## Discussion

In the here studied cohort of patients with rectal cancer, serum concentrations of TATI were lower than in the cohort investigated in Paper II, both in the full cohort and in rectal cancer patients. Interestingly, s-TATI levels remained unaffected by neoadjuvant RT and there was no significant difference between patients receiving neoadjuvant RT and untreated patients. Thus, the findings from this paper indicate that the lower levels of s-TATI observed in rectal cancer patients are not a result of neoadjuvant RT. RT has an ionizing effect on the tissue and free radicals are released causing cells to go into apoptosis [review][218]. RT has also been found to induce oxidative damage [219] and remodelling of the extracellular matrix by activation of e.g. PAI-1, MMP-2 and MMP-9 [113, 220]. Therefore, we hypothesized that RT might also affect levels of TATI in tumour tissue and/or serum, for which however no evidence was found in this study. While we are not aware of any other study on the effects of RT on TATI levels in tissue or serum, another study demonstrated that RT does not affect levels of TIMP-1 in rectal cancer tissue [114, 221]. However,

treatment with chemo-radiation in patients with locally advanced rectal cancer has been shown to increase levels of TIMP-1 in plasma [222].

Since it is becoming increasingly evident that CRC is a disease in with prognostic factors should be considered differently in women and men [223] the association of t-TATI with male gender in rectal cancer patients is of potential interest. Re-analysis of Cohort II from Paper I/II regarding the association of t-TATI and gender according to tumour location also revealed a correlation between male gender and higher t-TATI expression, and these associations should therefore be confirmed in future studies. However, no associations could be found between s-TATI concentrations and gender, neither in this cohort nor in the cohort from Paper II, and, throughout our studies, s-TATI was demonstrated to be a stronger indicator of poor prognosis than t-TATI. The disparate correlations of t-TATI and s-TATI with gender are not surprising, since this study confirmed the previously observed lack of an association between t-TATI and s-TATI. Hence, it is plausible to assume that TATI concentrations in tumour tissue and serum of CRC patients reflect different biological aspects of the disease.

Similar to Paper II, we found significant associations between s-TATI, but not t-TATI, and higher age. In this study, we also had the opportunity to investigate the association of TATI and s-creatinine, which was not possible in Paper II. S-creatinine concentrations were not significantly associated with age (Spearman's correlation = 0.230, P = 0.094, data not shown in Paper III) but we found significant associations between s-TATI, but not t-TATI, and s-creatinine levels both before RT and at surgery, but not at follow-up. Although the number of patients in this study was too small to allow for adjustment of s-creatinine in the survival analyses, the results indicate that s-creatinine levels should be taken into consideration in further, validatory studies related to the prognostic role of TATI in CRC.

The fact that t-TATI was only prognostic as assessed in the surgically obtained tissue, but not in the biopsy material, is most likely due to the small sample size and rather fragmented nature of the biopsies.

The findings of elevated s-TATI levels post-surgery in patients treated with short-term RT are in line with previous findings, and the role of TATI as an acute phase reactant [148]. The reason for s-TATI levels not being elevated in patients treated with long-term RT is less evident, but could be explained by the longer period of recovery before surgery. Notably, the distribution of clinicopathological characteristics was similar among patients receiving short-term and long-term RT, and no patients had received neoadjuvant chemotherapy.

# Paper IV

## Aims

In Paper IV, we investigated whether the in vitro observed interaction of TATI and EGFR might be reflected in the molecular pathology of human CRC and influence clinical outcome. For this purpose, we examined the associations of t-TATI and s-TATI with immunohistochemical expression of EGFR, selected downstream effector proteins pERK1/2 and pSTAT3, and *KRAS* mutation status in tumours from the cohort studied in Papers I and II. In addition, we examined the modifying effect of the investigative parameters on the prognostic value of t-TATI and s-TATI. For this purpose, immunohistochemical expression of EGFR, pERK1/2 and pSTAT3 was analysed on TMAs, and *KRAS* mutation status was analysed using pyrosequencing assay on a subset of fresh-frozen tumour samples (n = 210).

## Summary of results

No significant associations were found between t-TATI, s-TATI, EGFR, pERK1/2 and pSTAT3 expression. High t-TATI expression was significantly associated with *KRAS* mutations (R = 0.197, P = 0.005). Apart from an inverse correlation between pERK1/2 and M-stage (R = -0.165, P = 0.007), there were no significant associations between investigative markers and clinicopathological characteristics. High pERK1/2 expression was significantly associated with a shorter DFS (HR = 1.56 95% CI; 1.09-2.28), and high pSTAT3 expression with a shorter DFS (HR = 1.48 95% CI; 1.04-2.11) and TTR (HR = 2.00 95% CI; 1.10-3.65).

These associations remained significant in multivariable analysis for pERK1/2 (HR = 1.50, 95% CI 1.00-2.19 for DFS) and pSTAT3, (HR = 1.50, 95% CI; 1.00-2.26 for DFS and HR = 2.08, 95% CI; 1.04-4.18 for TTR).

Increased EGFR expression was significantly associated with a shorter TTR in multivariable analysis (HR = 1.99, 95% CI; 1.04-3.83, P = 0.039) but not univariable analysis. *KRAS* mutation was significantly associated with a shorter OS (HR = 1.54 95% CI; 1.06-2.24). Compared with patients that had EGFR negative tumours and low s-TATI levels, patients with EGFR positive tumours and high s-TATI levels had a significantly reduced survival ($P_{\text{interaction}}$ = 0.004 for DFS, and $P_{\text{interaction}}$ = 0.029 for TTR and $P_{\text{interaction}}$ = 0.049 for OS).

## Discussion

Even though the results from this paper give some indications of a prognostic interaction between TATI and EGFR, they do not provide a straightforward translation of previous in vitro data. Most importantly, the prognostic interaction was only observed for s-TATI, not t-TATI, which does not support an autocrine induction of EGFR by TATI in human CRC in vivo. The mechanistic basis for the potential effects of s-TATI on the growth of EGFR expressing colorectal tumours is less evident. Speculatively, this effect could be exerted on circulating tumour cells, by influencing their capability of forming metastases. However, in contrast to t-TATI, we found no association between s-TATI and time to liver metastasis. On the other hand, the observation that TATI expression was significantly lower in lymph node metastases compared to primary tumours in the here investigated cohort could indicate that TATI is downregulated in metastatic CRC cells. This could, in turn, indicate that the effects of t-TATI on EGFR-mediated tumour growth are only evident in earlier stages of tumourigenesis. In this context, the finding of significantly higher t-TATI expression in *KRAS* mutated tumours is of potential interest, indicating differential roles of t-TATI in *KRAS* wild-type and mutant tumours, respectively, which also opens up for research into the potential role of TATI as a predictor of response to anti-EGFR targeted therapies.

Apart from the association between t-TATI and *KRAS* mutation status, neither s-TATI nor t-TATI was significantly associated with any of the investigative markers. However, the choice of surrogate markers for downstream EGFR signalling, i.e. ERK1/2 and pSTAT3, might not have been optimal, since it was more dependent on the availability of validated antibodies than on a particular biological hypothesis.

# Conclusions

We can with this thesis conclude that:

- TATI in serum is a strong independent predictor of an impaired survival in CRC patients.

- TATI expression in tumour tissue has some prognostic value in CRC patients, but this is less evident than for TATI in serum.

- There is no significant correlation between TATI concentrations in tumour tissue and serum.

- Concentration levels of TATI in serum and tumour tissue are not affected by neoadjuvant radiotherapy in patients with rectal cancer.

- High tumour-specific TATI expression is associated with *KRAS* mutation status.

- The prognostic value of TATI in serum, but not tumour tissue, might be modified by tumour-specific EGFR expression.

# Future perspectives

From the results presented in this thesis work, it is evident that s-TATI is a strong indicator of poor prognosis in CRC patients, while the prognostic value of t-TATI is much more uncertain. This, together with the lack of an association between t-TATI and s-TATI, indicate different mechanisms behind elevated levels of TATI in tumour tissue and serum. Therefore, future clinical studies should focus on validation of the prognostic ability of s-TATI, also in a prospective setting. Moreover, even though TATI is not a CRC specific marker, it would be of potential interest to investigate whether pre-diagnostic s-TATI levels predict overall risk of CRC, as well as the incidence of more or less aggressive subtypes of the disease. For this purpose, one way to go could be to analyse TATI concentrations in baseline serum samples from cases and matched controls in the Malmö Diet and Cancer Study.

Future functional studies on the role of TATI in CRC, not least in the context of EGFR signalling, should also be pursued. Along this line, it would also be of interest to investigate the role of TATI as a predictor of response to anti-EGFR targeted therapies, preferably by analysis of tumour and serum samples from CRC patients enrolled in randomized clinical trials.

Another interesting avenue of research would be to investigate the correlation of TATI protein and mRNA expression levels, and whether the latter have a prognostic value in CRC. A next step could then be to perform gene set enrichment analysis (GSEA), a computational method that determines whether a prior defined set of genes shows statistically significant, concordant differences between two biological states (e.g. phenotypes), in this case comparing tumour with the lowest and highest and TATI expression. By this approach, novel pathways and processes associated with TATI expression may be identified.

# Popularised summary in Swedish

## Bakgrund

Tjock -och ändtarmscancer, med samlingsnamnet kolorektalcancer, är den näst vanligaste tumörsjukdomen i västvärlden hos båda könen och i Sverige insjuknar runt 5000 människor årligen i sjukdomen. Äldre individer har en ökad benägenhet att drabbas av sjukdomen då det generellt tar lång tid för den normala slemhinnan i tarmen att omvandlas till tumör. Orsaken till kolorektalcancer är inte helt klarlagd men ärftlighet och livsstil spelar viktiga roller. Kirurgiskt avlägsnande av tumören är den viktigaste behandlingsformen och tumörens utbredning, lokalt och i kroppen, s.k. kliniskt stadium, är en av de viktigaste faktorerna för att bedöma patientens prognos. Patienter med sjukdom som inte spridit sig till lymfkörtlar eller andra organ har oftast en mycket god chans att överleva efter enbart ett kirurgiskt ingrepp, medan patienter med spridd sjukdom ofta behöver kompletterande behandling, t ex strålning eller cellgifter. Dödligheten i kolorektal cancer är dock fortfarande hög och tumörstadium är ett otillräckligt verktyg för att avgöra vilka patienter som behöver mer intensiv behandling och vilka som har god prognos med enbart kirurgisk behandling. Det är därför av yttersta vikt att identifiera s.k. biomarkörer, t ex proteiner som uttrycks i tumörerna, för att bättre kunna skilja mellan mer och mindre elakartade tumörformer.

## Innehåll

I denna avhandling har vi studerat hur nivåer av enzymet Tumour-Associated Trypsin Inhibitor (TATI) i tumörvävnad och i blod hos kolorektalcancerpatienter korrelerar till risken att få återfall och dö i sjukdomen. TATI är ett enzym som förekommer naturligt i tarmvävnad, där det motverkar kroppsegna spjälkande enzym, såsom trypsin. TATI produceras även i större mängder i bukspottskörteln, där det antas förhindra en för tidig aktivering av trypsin. Både trypsin och TATI finns i mindre mänger även i andra organ i kroppen, där de har olika funktioner, t ex vid sårläkning,

där TATI antas balansera trypsinets effekt. Förhöjda nivåer av TATI i blodet ses även vid olika inflammatoriska tillstånd och efter operation.

Förekomst av TATI i tumörvävnad och/eller blod har tidigare även visat sig vara kopplat till en sämre prognos vid andra tumörsjukdomar, såsom äggstockscancer, urinblåsecancer, levercancer och prostatacancer. Sambandet mellan TATI och prognos vid kolorektalcancer var dock inte känt sedan tidigare då detta avhandlingsarbete inleddes.

I det första delarbetet analyserade vi nivåerna av TATI i tumörvävnad med hjälp av s.k. immunhistokemi i tumörer från två olika studiepopulationer (=kohorter) av patienter med kolorektal cancer. Studieresultatet visade att höga nivåer av TATI i tumörerna var kopplat till en större risk att få återfall, särskilt i form av spridning till levern, och att dö i sjukdomen.

I nästa delarbete undersökte vi nivåerna av TATI i blod från patienter tillhörande den större kohorten från det första delarbetet, dels för att se hur dessa överensstämmer med tumörnivåerna av TATI och dels om de påverkar prognosen. Vi fann intressant nog inget samband mellan TATI-nivåerna i tumör och blod, men att de senare hade en mycket starkare koppling till risk att få återfall och dö i sjukdomen än tumörnivåerna. I detta delarbete fann vi också att patienter med ändtarmscancer (rektalcancer), av vilka de flesta hade fått strålbehandling före kirurgisk behandling, hade lägre nivåer av TATI i blodet än patienter med cancer i tjocktarmen (colon).

I det tredje delarbetet undersökte vi därför om strålbehandling har någon effekt på nivåer av TATI i tumör och/eller blod hos patienter med rektalcancer. För detta ändamål analyserade vi TATI i prover med normal vävnad, tumörvävnad samt blod som tagits vid olika tillfällen hos 53 patienter med rektalcancer som behandlats med lång eller kort strålning, eller enbart kirurgi. Resultatet visade att preoperativ strålning inte påverkar TATI-koncentrationen i vare sig blod eller tumörer. Däremot ökade TATI-nivåerna i blodet efter operation, vilket är i linje med tidigare fynd. Som bekräftelse på resultaten i delarbete 1 och 2 fann vi också en koppling mellan TATI-nivåer i såväl tumörvävnad som blod och kortare överlevnad.

TATI har i tidigare studier visat sig ha stora strukturella likheter med en för cancerceller viktig tillväxtfaktorreceptor, den s.k. Epidermal Growth Factor Receptor (EGFR), Därför undersökte vi i det sista delarbetet sambandet mellan TATI, EGFR och andra till EGFR kopplade signaleringsmolekyler, med särskilt fokus på huruvida deras inbördes relation påverkar den prognostiska betydelsen av TATI. Vi använde oss av immunhistokemiska analyser och mutationsanalys av tumörer från den större patientkohort som studerats i första och andra delarbetet. Vi fann en koppling mellan tumöruttryck av TATI och mutation i en viktig gen, *KRAS*, vars protein i sitt normala tillstånd verkar som en strömbrytare för signalöverföring från EGFR till cellkärnan.

Därutöver visade det sig att EGFR-uttryck i tumörerna förstärkte den prognostiska betydelsen av TATI-nivåer i blod, i det att patienter som hade höga TATI-nivåer i blodet och EGFR-uttryck i tumörerna, hade den sämsta prognosen. Något liknande samband sågs ej mellan EGFR- och TATI-uttryck i tumörerna.

Sammanfattningsvis visar vi i denna avhandling att höga nivåer av TATI, fr a i blod, är en viktig prognostisk biomarkör vid kolorektal cancer och en potentiellt användbar markör för förbättrad klinisk handläggning av patienter med denna cancerform.

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