Colorectal Cancer –
Evaluation of MMP as a prognostic marker and a model for peritoneal response

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"In the world of surgical oncology, biology is the King, selection of cases is Queen, and the technical aspects of the surgical procedures are the Princes and Princesses who frequently try to overthrow the King and the Queen"

- Blake Cady

To Botvid, Eskil and Ingrid

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ABSTRACT

Background: There are about 6600 patients diagnosed with colorectal cancer in Sweden each year. Survival rates vary with cancer stage at diagnosis. The main treatment is surgery together with, in some cases, oncological treatment. Matrix metalloproteinases (MMP) are deeply involved in the growth and spread of colorectal cancer tumours. The aim of this thesis was to validate the methodology of sample storing and the measurement of MMP concentrations and evaluating the prognostic value of MMP in colorectal cancer survival. Furthermore, an experimental model for studying human peritoneal surface, *ex vivo*, was validated. **Methods**: *Study I* – Blood samples were obtained from 65 patients and analysed for MMP in citrated plasma and serum. *Study II* – Plasma, tumour biopsies and healthy intestinal biopsies were investigated before and after long-term cryopreservation to assess MMP level stability. In *Study III* a cohort of 272 patients were followed for 10 years after colorectal cancer surgery and the association between cancer-specific survival and plasma MMP concentration was analysed. *Study IV* - An *ex vivo* model of human peritoneum as well as a model for cultured mesothelial cells were developed. The models were subjected to trauma before introduction of cancer cells and followed by microscopy. **Results**: MMP have higher concentrations in serum compared to plasma and the variation in concentration is greater in serum samples. MMP concentration in plasma remains at the same level even after a long time in cryopreservation, while tissue extract concentrations appear to increase during storage. A high plasma concentration of MMP-1 in patients with non-disseminated disease was linked to worse cancer-specific survival after colorectal cancer surgery. The mesothelial cell model as well as the peritoneal model remained viable for long periods of time, and introduced cancer cells seemed to adhere to the edges of the traumatised area. **Conclusion**: Plasma samples are superior to serum samples when measuring MMP concentrations in circulating blood. Plasma samples could be stored for a long time at -80°C without MMP degradation. MMP-1 concentration in plasma in patients treated for colorectal cancer could have a prognostic value regarding cancer survival. Peritoneal models may be used to study colorectal cancer cell invasion and spread.

Keywords: Colorectal neoplasms; matrix metalloproteinases; colorectal surgery; survival; prognosis; peritoneum; peritoneal neoplasms.

Sammanfattning på svenska

Bakgrund

Tjock- och ändtarmscancer är den fjärde, respektive nionde, vanligaste cancerformen i Sverige och står tillsammans för ca 6 600 nya fall varje år. Den botande behandlingen är kirurgi där tumören opereras bort. I vissa fall används onkologisk behandling i form av cellgifter och/eller strålning som tillägg till kirurgi. Endast cirka 15 % av patienter som erhåller tilläggsbehandling i form av cellgifter har effekt av behandlingen. Överlevnaden i tjock och ändtarmscancer har förbättrats de senaste decennierna, patienter som drabbas har en beräknad 5-årsöverlevnad på ca 65 %. Överlevnaden varierar dock i stor grad, beroende på hur tumören har vuxit och om det finns dottertumörer i övriga organ i kroppen.

Matrix metalloproteinaser (MMP) är en grupp kroppsegna enzym som kan bryta ned beståndsdelar i det extracellulära rummet samt bidra till kärltillväxt och spridning av tjock- och ändtarmstumörer. Det har tidigare visats att det finns en korrelation av höga nivåer av MMPs i och kring tumören och överlevnad i cancersjukdom. Studier har också indikerat att höga nivåer av MMPs i blodplasma och serum kan ha koppling till cancerspecifik överlevnad hos patienter med tjock- och ändtarmscancer.

Spridning till bukhinnan är en fruktad följd av avancerad tjock- och ändtarmscancer. Biologin kring tumörinvasion och spridning på bukhinnan är inte helt känd och en anledning kan vara att det hittills saknats experimentella modeller för att studera detta förlopp.

Målsättning

Syftet med denna avhandling har varit att validera metoderna för mätning och förvaring av biologiska prov avseende olika typer av MMPs. Vidare har målsättningen varit att undersöka huruvida vissa MMPs, mätt i cirkulerande blod kan vara av prognostisk betydelse för överlevnad i tjock- och ändtarmscancer. Ytterligare ett mål har varit att validera en experimentell modell för att kunna studera mänsklig bukhinnevävnad i laboratoriemiljö för att få bättre förståelse om tumörbiologin vid spridning till bukhinnan.

Metod och resultat

I *Delarbete I* jämfördes nivåer av MMPs i blodplasma respektive serum hos 65 patienter. Vid analysen observerades att MMP-nivåer mätta i serumprover hade en större variation och bedömdes vara mer osäkra jämfört med plasmaprover. *Delarbete II* fokuserade på lagring av blod och vävnadsprover. Koncentration av MMPs mättes i plasmaprover, i tumörvävnad och tarmvävnad utan tumörväxt. Efter nio respektive 12 år gjordes nya mätningar på samma prover vilka visade att plasmaproverna var stabila och MMP-koncentrationen inte hade ändrat sig under lagringstiden. MMP-koncentrationerna ökade däremot under lagringstiden i såväl tumör- som tarmvävnad. Plasmanivåer och dess relation till överlevnad vid tjock- och ändtarmssjukdom undersöktes i *Delarbete III*. Nivåer av MMP i blodplasma undersöktes hos 272 patienter som opererades för tjock- eller ändtarmscancer vid Sahlgrenska Universitetssjukhuset/Östra 1999-2004. Plasmanivåerna jämfördes sedan med överlevnadsdata. Patienter med ett högt värde av MMP-1 i plasma hade kortare cancerspecifik överlevnad. Slutsatsen drogs att MMP-1 mätt i blodplasma sannolikt har ett prognostiskt värde och kan vara värdefullt vid uppföljning och behandling av patienter som drabbats av tjock- eller ändtarmscancer. I *Delarbete IV* validerades två experimentella modeller. I den ena modellen används odlade celler från bukhinnan och dessa undersöktes avseende påverkan från ett artificiellt kirurgiskt trauma. Det odlade cellagret skadades på ett standardiserat sätt och cellerna kunde sedan monitoreras genom mikroskopi. När humana cancerceller tillsattes noterades att cancercellerna verkade ha en tendens att fästa vid det skadade området. Detsamma gällde den andra modellen där bukhinna togs från patienter som genomgick kirurgi. Bukhinnan kunde sedan hållas viabel och studerades avseende påverkan av kirurgiskt trauma och tillsatta cancerceller. Modellerna kan ha stor betydelse i framtida studier för att ytterligare förstå biologin vid spridning av cancerceller från tjock- och ändtarm till bukhinnan.

Slutsatser
- Nivåer av cirkulerande MMPs bör mätas i blodplasma.
- Plasmanivåer av MMPs är stabila, efter långvarig förvaring.
- MMP-nivåer i vävnadsprover ter sig ej vara stabila över tid.
- Koncentrationen av MMP-1 i plasma hos patienter med tjock- eller ändtarmscancer kan ha ett prognostiskt värde avseende överlevnad.
- Modellen för att studera odlade celler och hel bukhinna fungerar och kan vara av stort värde i kommande studier av avancerad tjock- och ändtarmscancer.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Jonsson A, Hjalmarsson C, Falk P, Ivarsson ML. Levels of matrix metalloproteinases differs in plasma and serum – aspects regarding analysis of biological markers in cancer. Br J Cancer. 2016 Sep 6;115(6):703-706.

II. Jonsson A, Hjalmarsson C, Falk P, Ivarsson ML. Stability of matrix metalloproteinase-9 as biological marker in colorectal cancer. Med Oncol. 2018 Mar 9;35(4):50

III. Jonsson A, Falk P, Angenete E, Hjalmarsson C, Ivarsson ML. Plasma MMP-1 expression as a prognostic factor in colorectal cancer. Submitted 2019.

IV. Falk P, Jonsson A, Swartling T, Ivarsson ML. Colorectal Cancer Cells Adhere to Traumatized Peritoneal Tissue in Clusters, An Experimental Study. J Inves Surg. 2018 Aug:31(4):349-356.

Content

Abbreviations

AJCC	American Joint Committee on Cancer
BM	Basement membrane
CEA	Carcinoembryonic antigen
CI	Confidence interval
CRC	Colorectal cancer
CRS	Cytoreductive surgery
CSS	Cancer specific survival
CV	Coefficients of variability
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
fHB	Faecal haemoglobin
HIPEC	Hyperthermic intraperitoneal chemotherapy
HR	Hazard ratio
MMP	Matrix metalloproteinases
MSI	Microsatellite instability
PC	Peritoneal carcinomatosis
PG	Proteoglycans
ROC	Receiver operating characteristic
TIMP	Tissue inhibitor of metalloproteinase
TME	Total mesorectum excision
TNM	Tumour-Node-Metastasis
TS	Thymidylate synthase
UICC	The Union for International Cancer Control
VEGF	Vascular endothelial growth factor
5-FU	Fluorouracil

Introduction

Colorectal Cancer

Almost 3000 years ago, the natural course of a growing disease with intestinal origin was described as a treat to Joram, King of Judah by the prophet Elijah[1].

> *"And you yourself will have a severe sickness with a disease of your bowels, until your bowels come out because of the disease, day by day... The lord struck him in his bowels with an incurable disease. In the course of time, at the end of two years, his bowels came out because of the disease, and he died in great agony."*

Since then, the science of medicine has evolved, several milestones have been reached and colorectal cancer is no longer incurable. Tumours of the colorectal tract were later described by the Greek physician Aulus Celsus (25 BC-AD 50)[2]. Thereafter it took almost 2000 years until the first report of colon cancer surgery in 1823. It remained a rather unusual procedure; only ten colon resections had been reported by 1880. By the late 19th century, numbers of segmental resection of the colon had increased and the mortality rate began to decline[3]. The first procedure with radical intent for rectal cancer was performed in 1907[4].

Surgical techniques have changed dramatically over time. In 1908, the distribution of lymph nodes following the arterial blood supply of the colon was discovered, and the principal of resection of these vessels and lymph nodes was proposed as part of surgery for colon and rectal cancer[5]. Surgery for rectal cancer further evolved during the 20th century when several approaches to radical surgery were developed. A major advance was made by Dr R J Heald with the development of the total mesorectum excision (TEM) technique in the early 1980s[6]. Further progress in the treatment of colorectal cancer was made as a result of the introduction of adjuvant and neoadjuvant therapies. The use of preoperative radiotherapy in selected rectal cancer cases in the 1990s increased survival and decreased local recurrence rates even more[7]. Chemotherapy has also made great advances since its introduction in the middle of the 20th century. The first reported chemotherapy agent, 5-FU is still in use, but there are now

1

numerous new agents available, including targeting therapies with monoclonal antibodies[8].

Incidence and Survival

Globally there are around 1.2 million new colorectal cancer (CRC) cases each year, predominantly in developed countries. However an 80 % increase in CRC cases is estimated over the coming two decades, mostly in developing countries[9]. Colorectal cancer is the fourth most common cause of death from cancer worldwide and the third most common form of cancer, accounting for 9.7 % of all cancer cases in the world[10]. In Sweden, about 6600 CRC cases are reported each year[11]. The median age at which colon cancer is diagnosed in Sweden is 74.1 years. There is equal distribution between genders[12]. Rectal cancer, however, is more common in men with a ratio of 1.5:1, with a median age at diagnosis of 71 years[13]. The incidence of colon cancer has been increasing over the last 40 years while the incidence of rectal cancer has remained stable.

Overall survival has improved over the last 20-30 years. This is due to improved surgical technique, including metastasis surgery of the liver, lungs and peritoneal surface, as well as use of new oncological treatments and enhanced staging procedures[14-16].

Figure 1a. *Colon Cancer, 1980-2016. New cases per 100 000 inhabitants. Age adjusted. Sweden. Cancerregistret, Socialstyrelsen.*

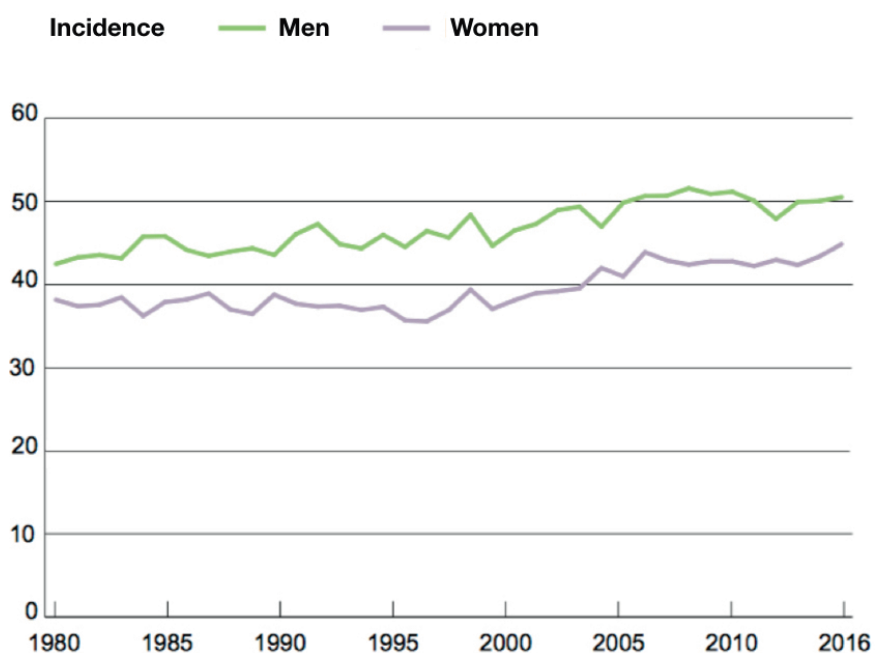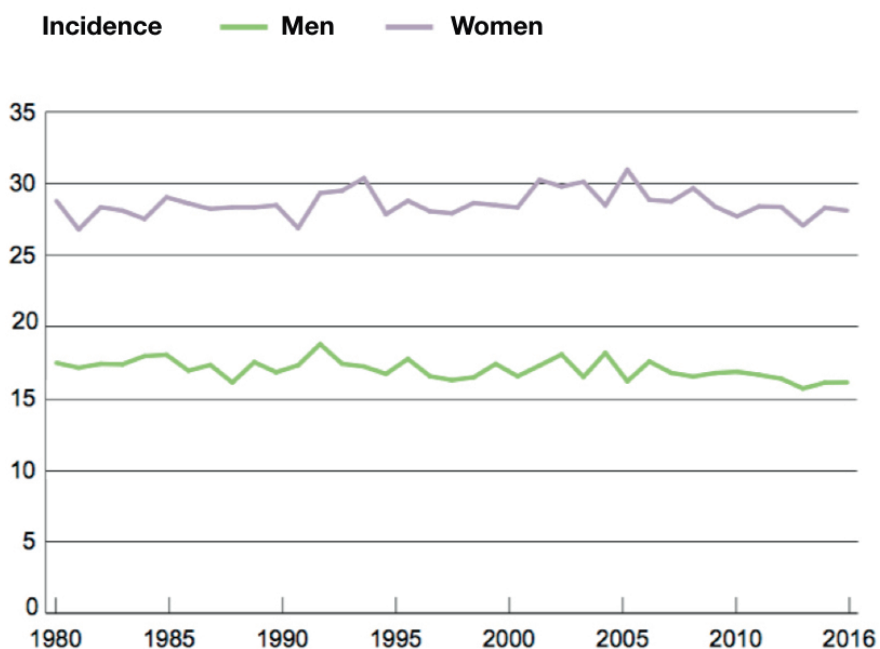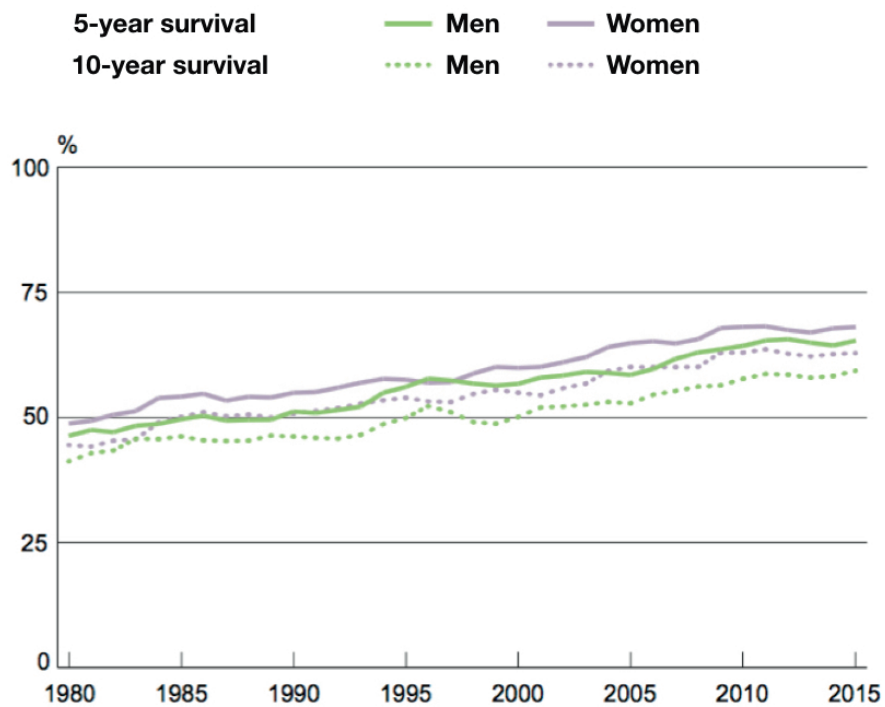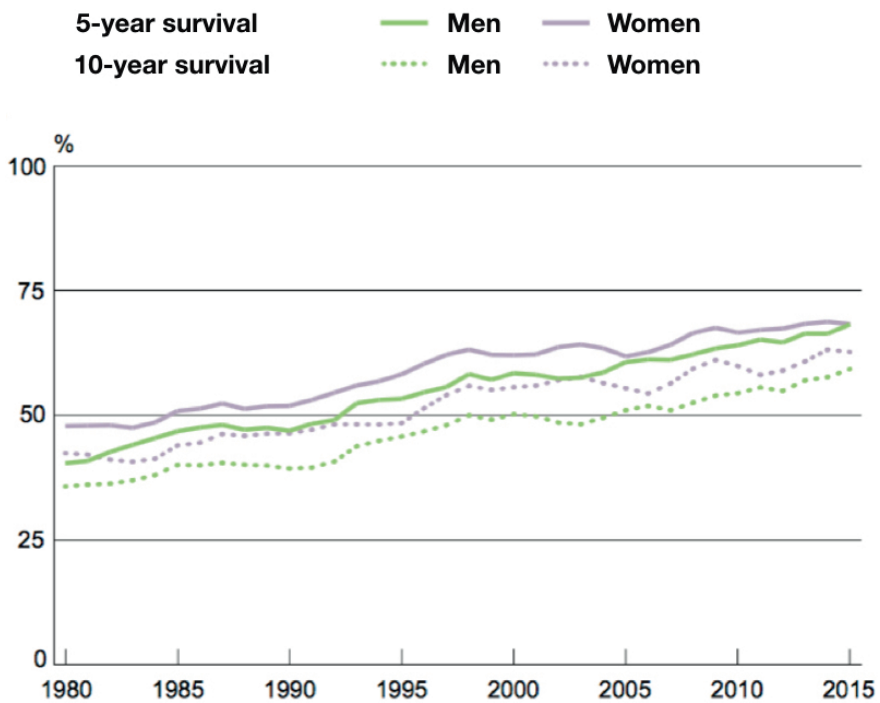***Figure 1b.*** *Rectal Cancer, 1980-2016. New cases per 100 000 inhabitants. Age adjusted. Sweden. Cancerregistret, Socialstyrelsen.*

Figure 2a. *Colon Cancer, 1980-2015.*
Relative survival. Sweden. Cancerregistret,
Socialstyrelsen.

Figure 2b. *Rectal Cancer, 1980-2015.*
Relative survival. Sweden. Cancerregistret,
Socialstyrelsen.

4

Epidemiology

There are several known risk factors for developing colorectal cancer, such as obesity, smoking, alcohol and red meat. On the other hand there are reasons to believe that physical activity and diets high in starch decrease the risk for colorectal cancer. Inflammatory bowel disease has traditionally been thought to be a strong risk factor for the development of CRC. However, the risk is probably not as high as previously reported. Cumulative risk for CRC in patients with IBD is 1 %, 2 % and 5 % after 10, 20 and > 20 years disease duration respectively[17-20].

Approximately 5 % of all CRC patients have a specific inherited syndrome, the most common being the Lynch syndrome where 50 % - 80 % of individuals with this condition develop CRC during their lifetime. Individuals with an autosomal dominant mutation that leads to familial adenomatous polyposis (FAP), have a 100 % risk of developing CRC, whereas a less severe attenuated form of FAP has a 69 % lifetime risk for developing CRC[21].

Staging

Colorectal tumour staging has a long history, with the Dukes classification first described in 1932. This classification ranges from Dukes A to Dukes D and is based on the anatomically characteristics of the tumour. This staging system was further refined to include the in-wall spread of tumour cells in 1954[22].

The currently used TNM classification was developed by the American Joint Committee on Cancer (AJCC). As to date, the 7th edition is used and the 8th edition is on its way. Even the TNM system is based on the anatomical characteristics of the tumour, including tumour spread throughout the bowel wall, the spread to lymph nodes and the presence of metastatic disease[23].

In this thesis the 6th edition of The Union for International Cancer Control TNM classification system and staging (UICC stage) of cancer disease was used. Involvement of the tumour in the bowel wall is represented by the T stage: T1 tumours have invaded the submucosa; T2 tumours have invaded the muscularis propria; T3 tumours have passed through the muscularis propria into the subserosa, or into the pericolic/perirectal tissue; and T4 tumours have invaded through the visceral peritoneum or into other organs. The N stage represents lymph node involvement: N0 indicates that there is no metastasis to regional

lymph nodes; if there are one to three lymph nodes with metastasis, the tumour is classified as N1; and if there are more than three lymph nodes involved it is classified as N2. The M stage represents distant metastasis where M0 implies no distant metastasis and M1 implies that distant metastasis are present[24]. The different TNM stages are visualised in *Figure 3*.
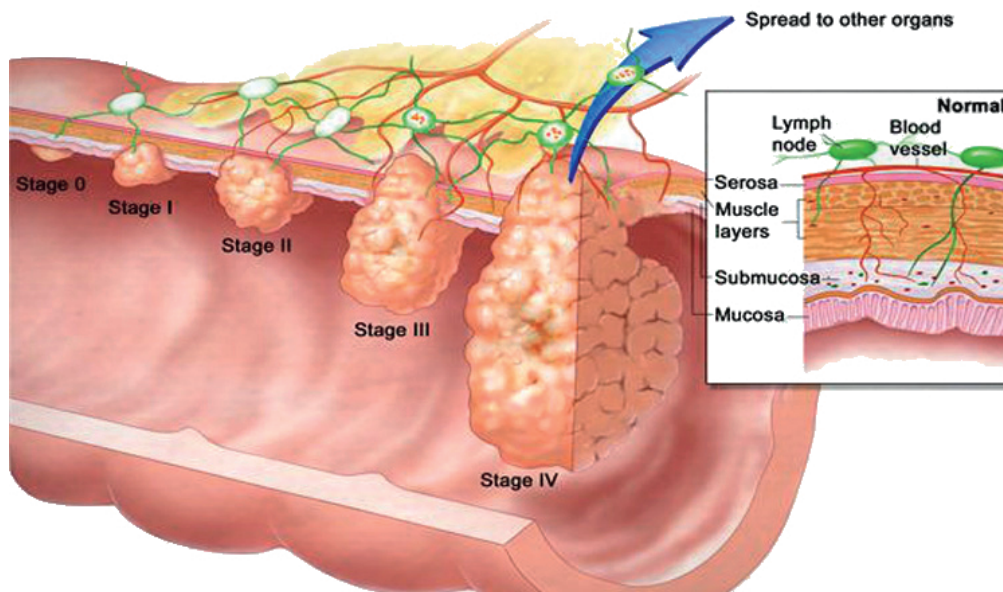
Figure 3.*TNM stages of colorectal cancer.*
National Cancer Iinstitute. With permission

UICC stages and corresponding 5-year cancer specific survival rates are shown in *Table 1*[25, 26].

Table 1. UICC stages and Cancer specific survival rates.				
UICC Stage	T stage	N stage	M stage	5-year CSS
I	T1 or T2	N0	M0	92.5 %
IIa	T3	N0	M0	83.6 % - 84.7 %
IIb	T4	N0	M0	69.1 % - 72.2 %
IIIa	T1 or T2	N1	M0	83.1 % - 83.4 %
IIIb	T3 or T4	N1	M0	64.1 % - 64.4 %
IIIc	Any T	N2	M0	44.3 % - 44.8 %
IV	Any T	Any N	M1	8.1 % - 10.4 %
Abbreviation: UICC = Union for International Cancer control; CSS = Cancer Specific Survival.				

Treatment

Surgery

Surgery is the cornerstone of colorectal cancer treatment. The principle of colon cancer surgery is a wide resection of the bowel to achieve a sufficient tumour-free margin. To warrant that the lymphatic drainage is included in the specimen the embryological planes should be followed together with a standardized pattern of blood vessel resection[27, 28].

Location of the tumour is fundamental when deciding on the extent of surgical resection. A tumour in the ascending colon is treated with right-sided hemi-colectomy where the ascending colon and mesocolon are resected, including proximal ligation of the corresponding blood vessels. A tumour in the proximal transverse colon is treated with an extended right-sided hemicolectomy. Tumours of the distal transverse colon as well as the descending colon are treated with left-sided hemicolectomy. Surgery is carried out in a similar fashion with resection of the corresponding mesocolon and vessels. However there is no consensus regarding exactly were the arterial blood supply should be ligated[29-33].

Rectal tumours are surgically removed according to the TME procedure where all the mesorectum, including the mesorectal fascia and the lymph nodes, draining the rectal tumour are resected.

Neoadjuvant Therapy In Rectal Cancer

For mid- and high-situated lymph node-negative T1- T3 rectal tumours, TME surgery is usually performed without any neoadjuvant treatment. However, more unfavourable rectal tumours with lymph node involvement, low-situated tumours, tumours that involve the mesorectal fascia, or tumours showing signs of extramural vascular invasion are treated with neoadjuvant radiation and/or chemotherapy to decrease the risk of local recurrence and/or to facilitate radical surgery[6, 34-37].

Neoadjuvant Therapy in Colon Cancer

There are several on-going studies aimed to evaluate if neoadjuvant chemotherapy has a place in cases of more advanced colon cancer, i.e. high-risk Stages II and III[38-40]. Reliable clinical staging (cTNM) is fundamental in the decision to use neoadjuvant chemotherapy. Lymph node status is assessed using computed tomography. The accuracy of detecting pathological lymph nodes, i.e.

Stage III colon cancer, has been reported to have a sensitivity of 62 - 88 % and a specificity of 55 – 70 %[41, 42].

Adjuvant Therapy

Adjuvant chemotherapy is given to selected patients depending on the TNM status. It is standard treatment for patients with Stage III disease, given they are fit enough to manage the systemic toxicity effects that follow this treatment[43-45]. In clinical practice colon cancer and rectal cancer are treated using the same adjuvant chemotherapy protocol, though the benefits of adjuvant chemotherapy in rectal cancer are not as well documented as in colon cancer. Despite updated TNM staging procedures and numerous attempts to find prognostic and predictive models, controversy still exists about how to treat patients with advanced Stage II CRC following surgery. There are no rigid indications, but selected Stage II patients with risk factors are normally recommended adjuvant chemotherapy[46].

Some Suggested Prognostic Markers

Several attempts have been made to achieve a better prognostic model for the outcome of CRC patients. The TNM system is now in its 7th edition, but there is still concern about its reliability in predicting prognosis[47-49]. Furthermore, the TNM system is based on the anatomic and pathological findings in the specimen resected in the operating theatre, making the classification system not useful in the preoperative work up of the patient[23]. The TNM system includes the metastatic involvement of regional lymph nodes. Traditionally 12 harvested lymph nodes have been recommended to enable staging and exclude metastatic disease, though a greater yield of cancer-free lymph nodes may increase the chance of survival[50].

There are many reasons to refine prognostic models for patients with CRC, including individualisation of treatment and follow-up programmes.

CEA

Circulating biomarkers of colorectal cancer have been extensively studied, but as yet only one biomarker has been widely used *i.e.* Carcinoembryonic Antigen (CEA). CEA is a normal product of the cell but is over-expressed in colon and rectum adenocarcinoma cells, as well as in other forms of adenocarcinoma[51]. CEA is mainly used as a monitoring biomarker following colorectal tumour resection, even though its sensitivity and specificity are questioned[52-54]. There

are studies, however, supporting the use of serum CEA levels as a diagnostic tool in CRC and colorectal adenomas[55]. Furthermore, CEA levels have been showed to increase with tumour stage and could be used to identify patients with a greater disease burden[56].

Circulating Tumour DNA

Circulating tumour DNA (ctDNA) from CRC tumours may be found in serum and plasma. As with CEA, ctDNA levels increase in patients with colorectal cancer disease and fall significantly after CRC surgery. ctDNA levels correlate with tumour stage and increase with cancer recurrence so it could also e used as a monitoring marker after CRC surgery[57, 58]. ctDNA is of most useful in more advanced stages of CRC as earlier stages does not express enough ctDNA to be measured in circulated blood[59].

Thymidylate synthase

Thymidylate synthase (TS) is involved in the synthesis of dTTP which is essential for DNA synthesis. A high concentration of TS correlates with poor CRC survival, local recurrence and distant metastasis[60]. Furthermore, high TS expression is known to increase the risk of resistance to the widely used chemotherapy agent 5-FU since this targets TS[61, 62].

Microsatellite Instability (MSI)

MSI is the result of a faulty mismatch repair gene (MMR). MMR is responsible for genome stability by correcting base-base mismatches during DNA replication. Microsatellite instability (MSI) occurs in about 15% of all sporadic CRCs. MSI could result in several mutations resulting in over-expression of Transforming Growth Factor-β (TGF-β) and Insulin-like Growth Factor (IGF), which play a key part in tumour development[63, 64]. CRC tumours that are MSI-positive have a better prognosis than those that are Microsatellite Stable (MSS), with lower recurrence rates and better overall survival[65, 66].

Vascular Endothelial Growth Factor (VEGF)

VEGF is the main stimulator of angiogenesis in the body, and is also known to be the most important promoter of angiogenesis in CRC. Studies indicate that over-expression of VEGF in CRC tumours is linked with poor outcome[67, 68].

Colorectal Cancer Microenvironment

Colorectal cancer tumours result from a series of mutations in the genome of epithelial cells in the colon and rectum. The resulting alteration in the genome is called the "Hallmark of cancer"; inactivating suppressor genes and activating oncogenes, leading to[69];

- Rapid, uncontrolled growth due the tumour cell's independent ability to produce growth factors, as well as resisting antigrowth signals.
- Promotion of tissue invasion and metastasis by remodelling of the extracellular matrix (ECM).
- Limitless replication potential
- Promotion of angiogenesis
- Avoidance of apoptosis

An accumulation of mutations and epigenetic alteration in the normal epithelial cell paves the way for the transformation of normal colon epithelium, first to early adenoma, then to advanced adenoma and finally adenocarcinoma[70].

The Extracellular Matrix

The extracellular matrix consists of water, proteoglycans, glycoproteins and proteins. The structure of the ECM varies between organs. The ECM is not static but is constantly being remodelled. Collagen, elastin, fibronectins and laminins are the principal proteins of the ECM. Moreover the proteoglycans (PG) supply a hydrated gel in the extracellular surroundings providing hydration, buffering and stability. Schematic cross sections of the ECM are illustrated in *Figure 4*.

The ECM is bioactive and important in adhesion, migration, proliferation and survival of the cell, and therefore plays a most important role in the pathobiology of cancer[71-73]. The structure of the ECM surrounding a growing tumour is different to normal ECM. The malignant CRC cells as well as surrounding stromal cells induce pathways in the ECM leading to the stimulation of invasive adenocarcinoma[74].

Figure 4. *The extracellular matrix in normal, aged, wounded and tumour invaded tissue.*
Elsevier Inc. With permission.

In order to invade the ECM and promote tumour invasion and metastasis, the tumour cells first release factors that promote attachment to the matrix. Once attached, mediators promoting ECM degradation are released providing pathways for the tumour cells to migrate across the stroma[75].

Degradation and modification of proteins in the ECM is fundamental to colorectal cancer cell invasion. Several types of proteinases are known to be involved including serine-, cysteine-, aspartate-, and threonine proteinases as well as a family of proteinases called the matrix metalloproteinases (MMPs) that form the basis of this thesis[76].

The Peritoneal Surface

The peritoneum of an adult has an area of about 2 m^2. The peritoneal surface is divided between parietal peritoneum covering the abdominal wall and visceral peritoneum covering the visceral organs. The peritoneum consists of a mesothelial cell layer, covering a basement membrane (BM). Beneath the basement membrane lies the extracellular matrix of the peritoneum; the submesothelial layer[77].

Matrix Metalloproteinases

Matrix metalloproteinases form a group of enzymes that are capable of degrading proteins such as collagens, laminins, fibronectins and proteoglycans in the ECM. MMPs are zinc-dependent proteolytic enzymes containing an N-terminal signal peptide, a pro-domain and a catalytic domain where the zinc ion is located. MMPs are either bound to the cell membrane or secreted. Different MMPs have different substrates and are divided into collagenases, gelatinases, membrane type, stromelysins and matrilysins[78, 79].

MMP regulation is controlled by several mechanisms, including transcription, activation and inhibition. Matrix metalloproteinases are at first expressed in an inactive pro-form. Already activated MMPs and serine proteinases can activate pro-MMP[80]. Inhibition of active MMP is achieved by Tissue Inhibitors of Metalloproteinases (TIMP), and α-2macroglobulin[81].

MMPs´ Role in Cancer Invasion

MMPs can be synthesised by tumour cells but the major source of active MMP is non-malignant stromal cells such as endothelial and inflammatory cells around the tumour. MMP activity and expression is increased in many types of cancer[82], not only altering the environment around the tumour but also directly affecting tumour growth at the cellular level.

Remodelling of the ECM and changes in cellular adhesion are fundamental to tumour invasion and dissemination. Proteolysis of the ECM by MMPs enables tumour invasion. Cell to cell adhesion is reduced by cleavage of E-cadherin by MMP-3 and MMP-7, facilitating migration[83]. MMP-1, MMP-8 and MMP-13 primarily degrade collagens (i.e collagen Types I, II and III) that are key components of the intestinal stoma, while the basement membrane primarily consists of collagen Type IV. MMP-2, MMP-7 and MMP-9 are matrix metalloproteinases that have collagen Type IV as substrate[84].

Cancer cells acquire the capacity to unregulated growth partly by becoming self-sufficient in growth-factors as well as being resistant to antigrowth signals. TGF-β is an important factor in the regulation of tumour growth. In normal non-malignant cells TGF-β act as a tumour-suppressive cytokine but is in the malignant context this factor is used by the tumour to achieve invasion and metastasis by influencing the surrounding stromal cells. The malignant cell also develops resistance to the antiproliferation properties of TGF-β. Several MMPs such as MMP-2 and MMP-9 are able to activate TGF-β by converting it from its inactive pro-form and thereby have a tumour-promoting effect[85, 86]. A central part of tumour development is tumour angiogenesis. MMPs such as MMP-3, MMP-7 and MMP-9 play a major role by regulating the local level of active VEGF stimulating angiogenesis and thereby providing the tumour with sufficient blood supply and promoting systemic spread[87]. MMPs are crucial in pre-metastatic niche formation through extracellular remodelling. Increased MMP concentrations contribute to the recruitment of bone marrow-derived cells, and the release of MMPs increases vascular permeability[88]. Some of the actions of MMPs in tumour growth and metastatic spread are shown in *Figure 5*.
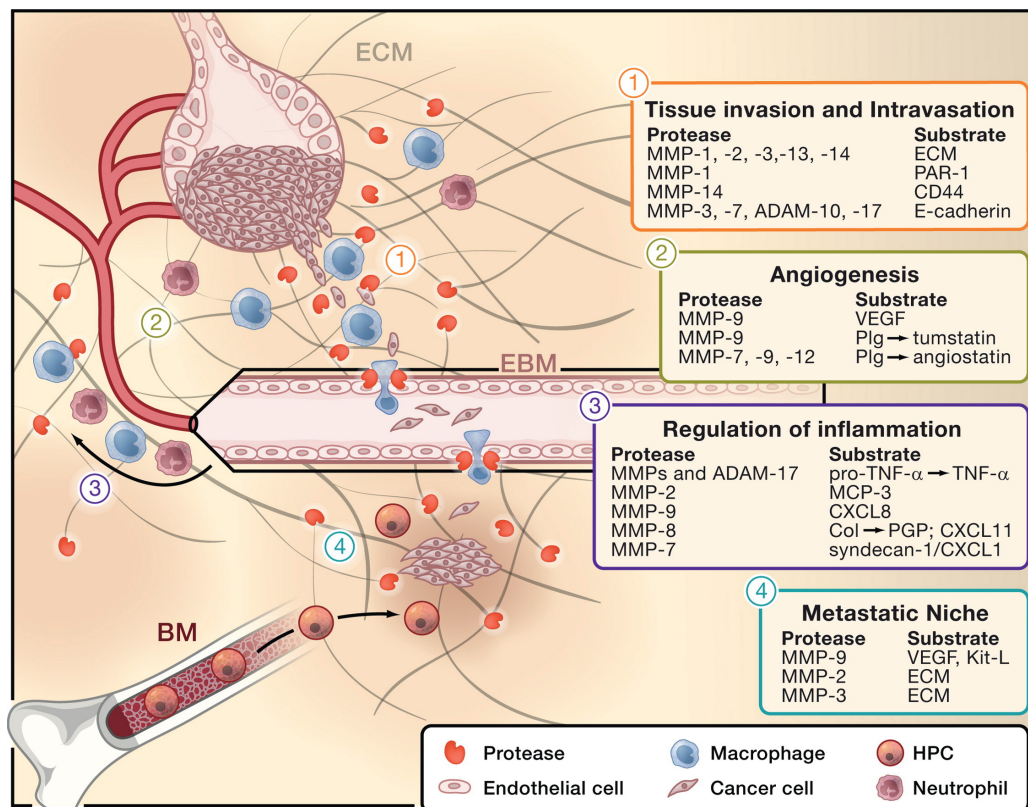
Figure 5. *MMPs role in cancer invasion. Invasion of the tumour and cellmigration into blood vessels by ECM remodeling and downregulation of cellular adhesion (1). Angiogenesis is triggered by VEGF release promoted by MMP-2 and MMP-9 (2). Inflammatory cells are recruited to the tumour environment by activating TNF-α (3).* Metastatic niche formation is facilitated by MMPs (4). Elsevier Inc. With permission.

Peritoneal Carcinomatosis

About 5-8% of patients with CRC have developed peritoneal carcinomatosis (PC) at the time of diagnosis, making the peritoneal surface the second most common site to metastatic disease, following liver metastasis in CRC[89, 90].

Approximately 5% of patients with PC from CRC are treated with cytoreductive surgery (CRS) combined with hyperthermic intraperitoneal chemotherapy (HIPEC). The median overall survival after this treatment is reported to be about 32-47 months, compared to systemic chemotherapy alone where the overall survival is about 10-17 months[16, 91].

The development of PC comprises five critical steps: 1. the release of cancer cells from the primary tumour into the abdominal cavity; 2. the transport of cells within the abdominal cavity, primarily by gravity but also gastrointestinal movement and the negative pressure caused by movement of the diaphragm; 3. thereafter the cancer cells need to adhere to the peritoneal surface where inflammatory mediators play a critical role; 4. Invasion of the submesothelial tissue then occurs by the degradation of ECM and further adhesion; 5. finally the invasion of the stroma beneath the mesothelial layer exposes cancer cells to the blood and lymphatic microcirculation[92]. The process is multifactorial but MMPs are thought to play a crucial role in these steps.

MMPs are key factors in the degradation of surrounding tumour stroma at the primary tumour site as well as mediating inflammatory responses, adhesion and degradation at the distant site, enabling tumour development on the peritoneal surface[83, 93-95]. It has been shown, both *in vitro* as well as in animal models, that selective MMP-inhibitors reduce PC and prolong survival[96].

Surgery for CRC could cause damage to the primary tumour leading to release of free cancer cells that may then adhere to the peritoneal surface. It is known that free cancer cells in the peritoneal cavity can lead to PC[97-99] Our knowledge of the biological interactions in tumour cell adherence to the peritoneal surface is still limited, one reason being the absence of a working human experimental model.

Matrix Metalloproteinases as Biomarkers In Colorectal Cancer

The activity and expression of MMPs is increased in many types of cancer and it is proposed that MMP levels measured in systemic blood circulation could be of prognostic and diagnostic value in cancer diseases[82]. Various MMPs, measured in blood have been suggested to indicate a possible potential as a diagnostic or prognostic marker in CRC[100-102].

Several studies have shown that MMP-9 levels are elevated in the tumour tissue and plasma of patients with CRC[102, 103] and have suggested that high MMP-9 levels may be used as a negative prognostic factor in CRC. Furthermore, patients with CRC show elevated MMP-9 levels in adjacent tumour-free mucosa and this could be used as a predictor for survival[104]. High MMP-7 concentration in the plasma of patients with CRC Stages I-IIB disease has been shown to correlate significantly with impaired survival[100]. MMP-2 in plasma increases with CRC

tumour stage and there is a significantly higher concentration in the plasma of CRC patients compared to healthy controls[102]. Furthermore, the serum concentration of MMP-8 in patients with colorectal cancer has been found to correlate with survival[105, 106]. Patients with CRC and a high expression of MMP-1 in tumour-free intestinal mucosa have poorer cancer-specific survival (CCS) compared to patients with low MMP-1 expression[107]. Faecal MMP-9 levels have recently gained attention as a promising diagnostic marker for CRC[108].

Measurement of Circulating MMP

There is a lack of consensus regarding the role of MMPs as prognostic or diagnostic markers of CRC when measured in circulating blood. Several studies have been performed with varying results. This may be due to the way blood samples have been prepared. Some studies on circulating MMPs have shown high levels in patients with CRC whereas others have not[109-111]. Circulating MMPs are analysed either in serum or in plasma. Serum is the liquid that remains from the portion of the blood when all cells are removed and the blood has clotted. In contrast with plasma that is the liquid that remains when clotting is prevented with a added anticoagulant. Analysis of serum samples for determination of circulating MMPs has previously been criticised[112, 113] since release of MMPs during the clotting process makes serum samples dependent on the time taken to analysis[114]. A comparison of the levels of certain MMPs in serum and plasma has not been fully explored.

To study cancer-related outcome in larger series, biological samples are usually collected and kept frozen until a sufficient number of samples, such as blood and tissue, have been collected and can be analysed in batches. The rationale behind this is to minimise inter-assay variation and to be more effective in the use of laboratory equipment. The stability of cryopreserved biomarkers, including MMPs in plasma and tissue samples, has been a matter of debate, and inappropriate handling of specimens may lead to degradation of biomarkers in cancer tissue[115, 116]. The long-term cryostability of MMPs in plasma and in centrifugal extracts of intestinal mucosa has not been fully investigated[117-119].

Aim

The objective of this thesis was to validate methods of measurement and storage of biological samples regarding MMP concentration, and to assess the role of MMPs as prognostic markers in colorectal cancer. Furthermore, to develop and validate an experimental model to investigate CRC cell adhesion in peritoneal carcinomatosis

The specific aims were to:

- Study I: Determine whether plasma or serum should be used when measuring MMPs in peripheral blood.

- Study II: Investigate the long-term stability of MMP-9 levels in cryopreserved citrated plasma, and centrifugal extracts of tumour tissue and tumour-free intestinal mucosa samples from patients with colorectal cancer.

- Study III: Evaluate if MMP-1, 2, 7, 8, and 9 measured in plasma could be used as a prognostic indicator for overall survival and cancer specific survival in patients treated for colorectal cancer.

- Study IV: To develop and validate an experimental model using human mesothelial cells and peritoneal tissue samples, to investigate CRC cell adhesion in peritoneal carcinomatosis.

Methods

Patients

Study I

Men and women 65 years-of-age and living in Kungsbacka municipality, Sweden, were asked to participate in a pilot study regarding CRC screening. There were 976 persons meeting the criteria of the pilot study. In all, 636 chose to participate and informed consent was obtained from all participants. The participants were screened for faecal haemoglobin (fHb) and 68 patients who were positive were examined by colonoscopy. In 65 of these patients there were no signs of CRC or adenoma. Serum and plasma samples from circulating blood were also obtained at the time of colonoscopy. Blood samples from 34 males and 31 women were included in Study I.

Study II

Thirty-six samples each of frozen plasma, tumour tissue and tumour-free large bowel tissue, respectively, were used in Study II. The samples were obtained from 47 patients during CRC surgery at Sahlgrenska University Hospital/Östra, Gothenburg, Sweden, between the years of 1999 and 2005. The samples were chosen to represent all UICC cancer stages.

Study III

A total of 331 patients operated on due to colorectal cancer between 1999 and 2005 at Sahlgrenska University Hospital/Östra, Gothenburg, Sweden formed the cohort of this study. Valid data were available for 272 patients at the end of follow-up.

All surgical resection specimens were sent for a pathological report according to the UICC TNM classification system, and staging of the cancer disease was made according to the 6th Edition of The American Joint Committee on Cancer. Neoadjuvant chemotherapy was not used. Adjuvant chemotherapy was offered according to protocol. All participating patients gave informed consent.

Patients and tumour characteristics were compared to all patients that underwent elective surgery for colorectal cancer at Sahlgrenska University Hospital/Östra,

Gothenburg, Sweden during the same period of time. The local database at Sahlgrenska University Hospital/Östra includes data of all patients that have been treated for colorectal cancer since 1999.

Study IV

Human mesothelial cells were used in Study IV. Primary cells were harvested from three patients undergoing non-septic benign open surgery. These cells were cultured to achieve established lines and then frozen in liquid nitrogen prior to the experimental set-ups. In the second part of Study IV, peritoneal tissue samples were obtained from four patients undergoing elective open surgery for a non-septic benign disease. Due to variation in the size of the human peritoneal tissue removed, different experimental set-ups were used for each patient. From four patients' tissue samples, seven set-ups were used in the study.

Data sources

In Study III, date of death and causes of death were obtained from the Swedish National Board of Health and Welfare Cause of Death Register. The register contains data for cause of death and covers all deaths in Sweden. The register has a coverage of 99.1 % and the underlying cause of death is recorded in 96 % of all deaths [120].

Sample preparation and storage

Study I

Venous blood samples were collected in a standardised way. Tubes without clot activators were used for serum samples and were stored for 30 minutes at room temperature (+20°C) before centrifugation at 10 000 g. The plasma samples were collected in citrate tubes and within five minutes centrifuged at 10 000 g at 20°C for 10 minutes. After centrifugation the supernatants were collected in small aliquots and frozen at -80°C pending analysis in batches.

Study II

In Study II, both blood and tissue samples were used. Blood samples were collected in citrate tubes after induction of anaesthesia. The tubes were centrifuged within five minutes at 10 000 g for 10 minutes at 20°C. Several aliquots were taken from each supernatant and were frozen at -80°C pending analysis.

Surgical biopsies where taken from the tumour and from tumour-free colon, approximately 10 cm from the tumour. Tissue samples were about 1 cm^2 in size. All samples were immediately frozen in liquid nitrogen in the operating theatre and further stored in -80°C until tissue extract preparation. In preparation for analysis, the samples were thawed, cut into pieces 40 mg in weight and homogenised in phosphate-buffered saline (PBS) buffer with 0.01% triton X-100 using 40 mg tissue per ml buffer. The homogenate was centrifuged at 10 000 g for three minutes and the supernatant collected in multiple aliquots and kept frozen at -80°C pending analysis.

Study III

During induction of anaesthesia, blood samples were collected in citrate tubes in a standardised manner and centrifuged at 10 000 g for ten minutes. The supernatants where frozen at -80 °C pending analysis. Surgical specimens were sent for pathology examination and staging of the tumour was made according to 6th Edition of The American Joint Committee on Cancer[121].

Study IV

In Study IV peritoneal tissue, harvested human mesothelial cells and commercial available Colo205 tumour cell lines were used. All handling in the culture laboratory was performed under aseptic and sterile conditions using a laminar air flow (LAF) bench (Holten, Ninolab, Kungsbacka, Sweden). Cultures were monitored regularly using inverted phase contrast microscopy and documented using the Axiovision system (Carl-Zeiss).

During surgery, peritoneal tissue samples were removed and placed in culture medium E199 (Sigma-Aldrich). The samples were immediately transported to the laboratory. The peritoneal samples were cut into 25 x 25 mm squares and mounted in our experimental model. The peritoneal squares were mounted between two acrylic rings with the mesothelial surface pointing upwards, see *Figure 6*. When the preparations were ready the samples were submerged into a cell culture dish where the culture medium was completely changed three times a week.
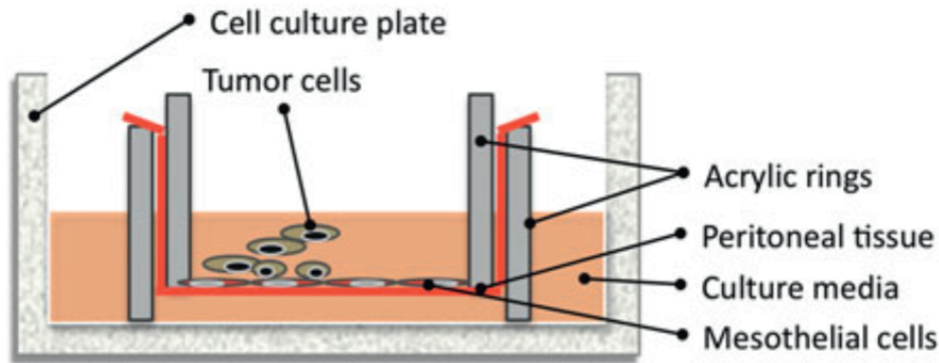
Figure 6. The human ex vivo tumour invasion model. Human peritoneum is placed between two acrylic rings during sterile conditions and placed in a cell culture incubator. The peritoneal membrane could then be monitored using inverted light microscope.

Frozen cell lines from human primary mesothelial cells previously harvested from the peritoneal fluid of patients undergoing surgery for non-septic benign disease. The cells were cultured in a cell incubator at 37°C and sub-cultured when confluent cell layers were observed. Growth medium was changed tree times a week.

A commercially available human colon cancer cell line, Colo205 was used. The cells were cultured and sub-cultured in a manner similar to the mesothelial cells.

Cell lines were initiated by thawing the frozen ampoule in a water bath at 37°C. The cells where then suspended in culture medium and centrifuged at 260 g for 10 minutes. The supernatant could then be discarded and the cell pellet transferred to a cell culture flask

Luminex xMAP

In Studies I and III the MMP measurements in serum and citrated plasma were carried out using Luminex xMap technology. Luminex xMAP uses small, colour-coded beads that are coated with an antibody designed to capture the specific analyte of interest. The sample, containing the desired analyte is then introduced in the assay (*Figure 7A*). A "sandwich" is thereafter created using an additional detection antibody aimed at the analyte. A fluorochrome (phycoerythrin) is added as substrate and binds to the detection antibody (*figure 7B*). The samples are then analysed with the Luminex xMap multi-assay technology (Bio-Plex 200, BIO-RAD, Sundbyberg, Sweden). The instrument detects each colour code of the beads with one laser and a second laser beam

determines the amount of signal from the added fluorochrome from each bead (*Figure 7C*). The level of MMP in the samples was calculated using a five parameter logistic (5-PL) standard curve[122]. Each sample was measured in duplicate.

A

STEP 1

Analyte

Capture Antibody

Luminex Bead

B

STEP 2

Detection Antibody

Streptavidin-PE

Streptavidin-PE
Biotin
Detection Antibody
Target Analyte
Capture Antibody

Luminex Bead

C

STEP 3

Polystyrene beads:

Streptavidin-PE
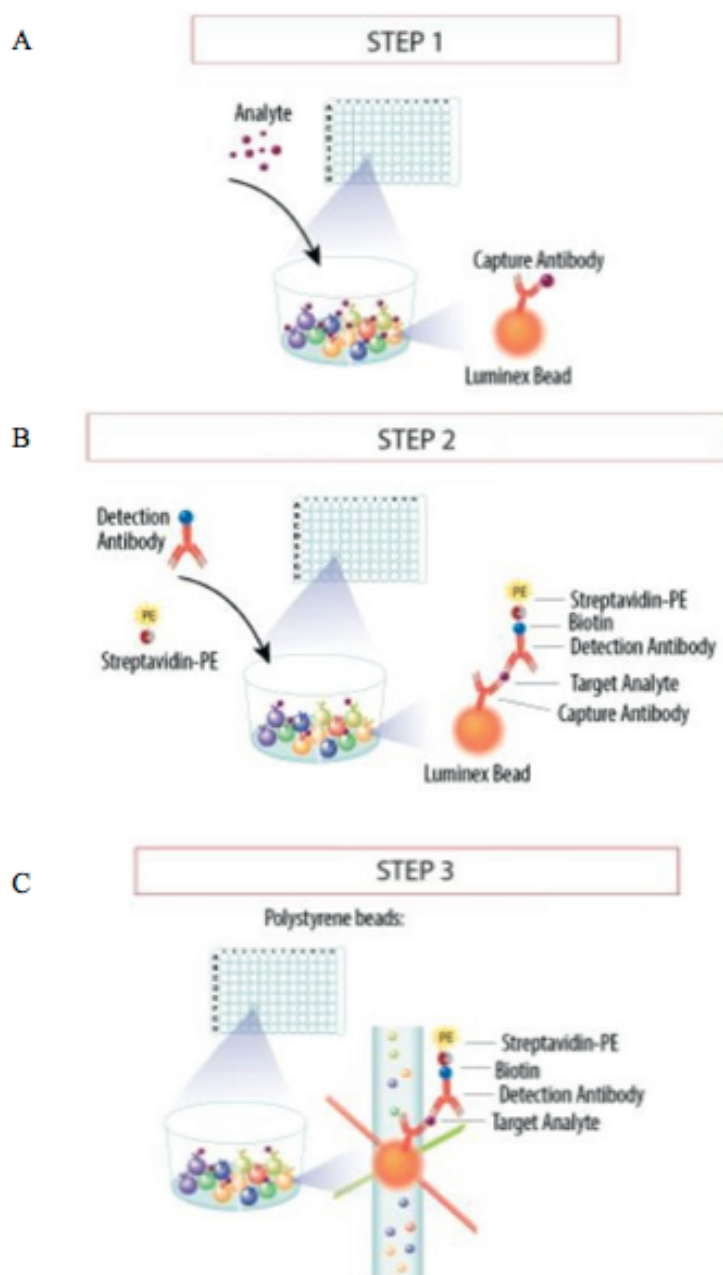Biotin
Detection Antibody
Target Analyte

Figure 7A-C. *The sample is introduced (A). A sandwich is created with a detection antibody (B). The instrument detects each color code and measure the amount of signal from the added flourochrome (C). R&D Systems, Bio-Techne, Minneapolis, USA. With permission.*

Levels of MMP-1, MMP-2, MMP-7, MMP-8, MMP-9 and MMP-13 were investigated in Studies I and III. Specific kits were purchased for these analyses. Their lower detection limit, intra- and interassay coefficients of variability (CV) are shown in *Table 2*.

Table 2. Detection limits and CVs of investigated MMPs

Analyte	Detection	LDL (pg/ml)	Intra-Assay CV (%)	Inter-Assay CV (%)
MMP-1	P, M, Ti	0.57	7.8 – 9.0	15.3 – 16.2
MMP-2	P, M	3.8	7.3 – 9.3	10.0 – 13.3
MMP-7	P, M, Ti	3.9	5.0 – 9.0	7.7 – 11.5
MMP-8	P, M, Ti	7.8	5.2 – 7.0	9.6 – 14.3
MMP-9	P, M, Ti	5.7	3.8 – 5.8	9.3 – 11.7
MMP-13	P, M, Ti	36.5	4.3 – 5.6	10.7 – 12.6

Abbrevations: LDL = Lower Detection Limit; CV = Coefficient of Variation;
MMP = Matrix metalloproteinase; P = Proform; M = Mature; Ti = MMP bound to Tissue Inhibitor of Metallproteinases.

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA technology was used in Study II for the measurement of MMP-9. The assay has 96 wells and is performed with the sandwich technique. Each well is precoated with the specific antibody that binds to MMP-9 when the sample is added. In the second step, the detection antibody is added and forms a sandwich complex. The substrate is then added, and after the addition of an acid solution to stop further reaction the plate is measured at a wavelength of 450 nm in a multi-well plate reader, see *Figure 8*. The colour intensity is proportional to the amount of detected MMP-9 in each sample[123].
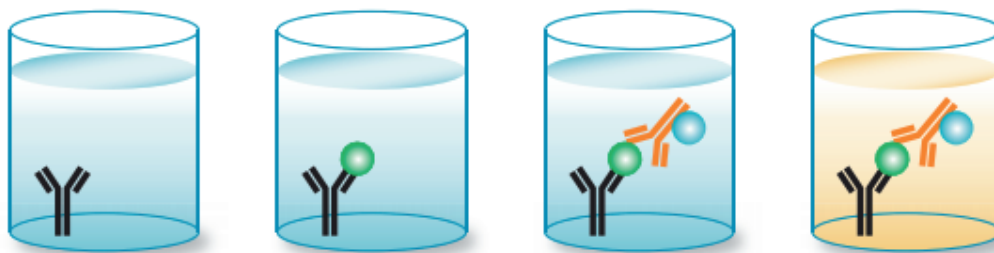

Figure 8. Direct sandwich ELISA Principle. A well is coated with a capture antibody, the analyte is added and thereafter a detection antibody. The substrate is added and the plate is measured in a multiwall-based plate reader. Bio-Rad Laboratories. With permission

The concentration of MMP-9 from plasma and the centrifuged homogenate was measured by absorbance using a standard curve and internal controls with known concentrations. The kit has an intra- and interassay CV of 4.9 - 5.5 % and 8.1 - 9.8 %, respectively, and the lower detection limit is 0.6 ng/mL. Each sample was measured in duplicate.

In vitro and *ex vivo* Trauma Models

Tumour Cells Labelling

In Study IV an *in vitro* as well as an *ex vivo* model were developed. Commercial available Colo205 cancer cells were used in the model. Before introduction of the tumour cells to each model they were labelled with a lipophilic tracer kit using fluorescent carbocyanine dye (Vybrant, Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA). This type of labelling is strong and remains visible for up to a week, and tumour cells remains fluorescent throughout sub-cultivation and the technique does not affect cell growth and proliferation. The labelling was performed before tumour cell introduction to our trauma model. The fluorochrome solution was added to the cell suspension and incubated for 30 minutes at 37 °C in the cell culture incubator. After centrifugation for 10 minutes at 260 g, the cells were resuspended in E199 growth medium.

Imitating Surgical Trauma in the Mesothelial
Cell Layer and Human Peritoneal Tissue Model

A culture plate was used for mesothelial cell cultivation. When a confluent layer of cells had developed, surgical trauma was imitated by scraping the monolayer with a sterile plastic pipette. The same procedure was performed with the peritoneal membrane in the *ex vivo* peritoneal model. To enable measurement of the wounded area, a calibrated size marker with software connected to the microscope was used (Axiovision, Carl Zeiss AG, Germany).

Introduction of Tumour Cells to the Trauma Models

The Colo205 cells were cultured in culture flasks, detached using trypsin/EDTA solution and resuspended in preheated culture medium. The cell pellets were produced by centrifugation at 260 g for 10 minutes and discarding the supernatant. To produce two different density suspensions, the cells were diluted and further resuspended in 5 ml E199 medium as 10 000 cells/mL and 100 000 cells/mL. The concentrations were verified by cell counting in a haemocytometer. The suspensions with low and high cell density were then

introduced to the traumatised cultured mesothelial cell layer model and the traumatised human peritoneal model respectively.

Monitoring and Photo Documentation

Cell cultures and the human peritoneal *ex vivo* models were monitored using inverted microscopy (Axiovision, Carl Zeiss AG, Germany). Photographic documentation was carried out at 0, 1, 2, 16, 24, 48, 96 and 192 hours after tumour cell introduction. Labelled tumour cells were photo documented using a mercury lamp together with a fluorescein isothiocyanate (FITC) and tetramethyl-rhodamine isothiocyanate (TRITC) filter set.

Statistics

The first three papers included in this thesis used statistical calculations. All tests were two-sided and $p < 0.05$ was considered significant. All calculations were carried out with IBM SPSS Statistics for Macintosh (Ver. 22.0, IBM Corp, Armonk, NY).

Paper I

In Paper I levels of MMP-1, MMP-8, MMP-9 were transformed with the natural logarithm to achieve normally distributed data and dependent t-test was used to compare means between plasma and serum samples concentrations. MMP-2 and MMP-7 were not normally distributed, even after transformation. Thus, the Wilcoxon signed rank test was used to compare medians. The Spearman rank correlation test was used to estimate correlation.

Paper II

In Paper II, MMP-9 concentrations were compared before and after cryopreservation. The Wilcoxon signed rank test was used to compare MMP-9 concentrations at baseline and after nine years of storage (plasma group) and at baseline and after twelve years of storage (tissue extract group). Correlations were estimated using the Spearman rank correlation test.

To visualise agreement between baseline and cryopreserved values, Bland-Altman plots were used. An estimated confidence interval of 95% of the mean difference of recovery rate was applied[124].

Paper III

Receiver operating characteristic (ROC) curves were used to divide MMP plasma levels between *high* and *low* levels. ROC curves were based on plasma MMP levels and data for survival, and the optimal cut-off value was estimated and used as a dichotomous variable in further analysis.

Kaplan-Meier curves were used to illustrate survival over time. Log-Rank tests were used to test for level of significance. Cox proportional hazard models were used to estimate the prognostic value of plasma MMP expression. Univariate analysis of UICC cancer stage, age, tumour site, the use of adjuvant treatment and MMP plasma level were executed. Variables with a p-value below 0.16 in univariate analysis were used in multivariable analysis[125]. Variance Inflation Factor (VIF) was calculated for each variable included in the multivariate analyses to test for multi-collinearity. Hazard ratios (HR) were displayed with a 95 % confidence interval (CI 95 %).

As we tested for several different MMPs in Study III there was an underlying risk of multiplicity. The more hypotheses tested the higher is the risk of "false" significance at the 5 % level. There are several models to correct for the risk of multiplicity. An often used procedure is the Bonferroni correction where the desired significance level is divided by the number of tested hypotheses. On the other hand, by choosing values using the Bonferroni correction or similar, there is some risk of missing actual differences. Study III evaluated the possible roles of several MMPs as prognostic factors in colorectal cancer, the study was more of an explorative nature aimed to find possible biomarkers, and with that intention multiplicity correction was not required. However, we did bear the risk of multiplicity in mind when interpreting our results and drawing conclusions.

Ethical Considerations

Informed consent was obtained from all participating patients. The studies were approved by the Ethics Committee at Lund University Hospital, Lund, Sweden and Sahlgrenska University Hospital, Gothenburg, Sweden.

Paper I: 2010-591, Paper II: LO19-99, Paper III: LO19-99 and 424-17, and Paper IV: Ö727-03 and Ö728-03

Results and Discussion

I. The Use of Citrated Plasma or Serum Samples in the Measurement of Systemic MMP Levels

MMP blood concentrations were measured in 65 patients, 65 years-of-age, with no sign of CRC at colonoscopy. Samples were taken as citrated plasma and serum. All samples were analysed in duplicate.

For all MMPs measured (MMP-1, MMP-2, MMP-7, MMP-8 and MMP-9), the levels in serum were found to be higher than those in plasma ($p < 0.01$). The concentrations of MMP-13 were found to be under the detection limit of the assay in all samples, and were thus not reported.

MMP-1 was 7.9 times higher in the serum group compared to the citrated plasma group ($p < 0.01$, n = 65), for MMP-2 the levels in serum were found to be 1.2 times higher ($p < 0.01$, n = 65). MMP-7 was 1.5 higher in the serum group, while MMP-8 and MMP-9 were found to be 4.7 and 5.6 times higher in serum compared to citrated plasma. There were no differences in MMP concentrations between male and female patients, in both the serum and citrated plasma groups ($p > 0.05$). Medians and interquartile ranges are reported in *Table 3*.

Table 3. Medians and interquartile ranges of MMP levels in plasma vs serum

	Plasma	Serum	p-value
MMP-1 ng/ml (IQR)	0.725 (0.921)	5.70 (6.50)	< 0.01
MMP-2 ng/ml (IQR)	288 (216)	340 (252)	< 0.01
MMP-7 ng/ml (IQR)	2.45 (2.31)	3.56 (2.66)	< 0.01
MMP-8 ng/ml (IQR)	1.82 (4.06)	8.47 (13.5)	< 0.01
MMP-9 ng/ml (IQR)	48.2 (64.7)	270 (254)	< 0.01
Abbreviations: IQR = Interquartile range; MMP = Matrix metalloproteinase			

Interquartile ranges were all greater in the serum group compared to the plasma, indicating a wider distribution. The results are visualised in boxplots according to *Figures 9A-E*.
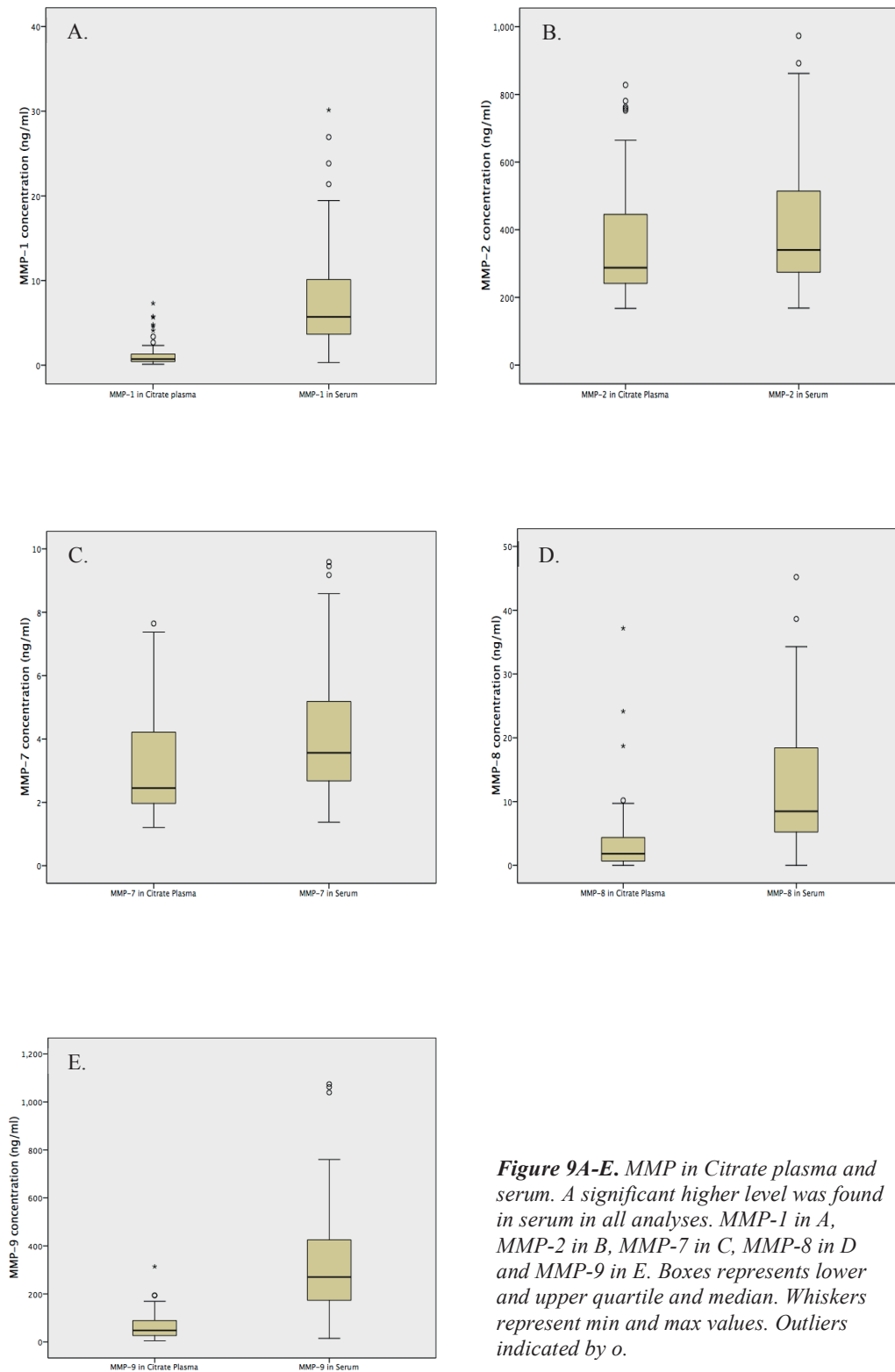
Figure 9A-E. *MMP in Citrate plasma and serum. A significant higher level was found in serum in all analyses. MMP-1 in A, MMP-2 in B, MMP-7 in C, MMP-8 in D and MMP-9 in E. Boxes represents lower and upper quartile and median. Whiskers represent min and max values. Outliers indicated by o.*

This study involved a large sample size and compared several MMPs in plasma and serum. Despite this, the material must be seen as restricted since it comprised patients of the same age. Furthermore, the patients could not be regarded as a "healthy" group since the only exclusion criterion in the study was sign of CRC at colonoscopy. It is known that MMP varies with age, and previous studies have reported gender differences[126, 127]. . The present study did not find any significant difference in MMP concentrations between males and females in the serum or plasma group. The difference between MMP levels in serum and plasma, however, was quite clear, regardless of age or sex.

Our results concur with previous work on MMP levels in plasma and serum. Previous studies have used fewer patients and/or analysed fewer forms of MMP[128, 129] some showing no significant difference between MMP levels in serum and in plasma. However, the results of the present study in this thesis were based on a larger sample size[113, 130]. One study looking at MMP-9 levels in plasma and serum of patients with gastric cancer gave different results; the authors found a significant difference in plasma MMP-9 levels in patients with known gastric cancer compared to healthy controls. However no difference was seen when serum MMP-9 levels were compared[131].

In Study I we used citrate as anticoagulant for the plasma samples. EDTA or heparin could have been used, but previous studies indicate that citrated plasma has less variability and is more stable during prolonged storage before centrifugation[112, 130].

In Paper I we report higher concentrations and wider distribution of MMP levels in serum compared to corresponding citrated plasma samples. The reason for the greater interquartile ranges and overall higher concentrations in serum is not fully understood. There might be release of proteases during the clotting process in the serum tube that the anticoagulant properties of citrate prevent[114]. The concentrations of several MMPs in serum tubes are known to increase as the time between venous sampling and centrifugation is prolonged. This could be due to MMPs in platelets, neutrophils and white blood cells being released during aggregation[132-134]. MMP-2 levels, however, are not greatly affected by the clotting process, which may explain our results where MMP-2 in serum did not differ as much from plasma[135].

Paper 1 concludes that the value of MMP levels measured in serum is limited due to greater variability and dependency on the time-to-analysis compared to levels analysed in plasma.

II. Reliability of Frozen Plasma and Tissue Samples When Measuring MMP Concentration

To validate our cryopreservation set-up, 36 samples from each plasma, tumour tissue extract and extract from intestinal tumour-free tissue were analysed for MMP-9. The samples from plasma were stored for nine years and all tissue samples were stored for 12 years. The samples were analysed at baseline *i.e.* within 24 months following surgery. Corresponding aliquots were then analysed after cryopreservation.

MMP-9 in plasma had a baseline median concentration of 9.9 ng/mL. The median level after 9 years of cryopreservation was 9.7 ng/mL. This difference is not significant according to the Wilcoxon signed rank test ($p > 0.05$).

Cryopreservation of tumour-free intestinal tissue extract showed an increase in concentration over storage time. MMP-9 at baseline was 7.1 ng/mL and after cryopreservation 8.1 ng/mL. When tested with Wilcoxon signed rank test the levels were found to differ significantly ($p < 0.05$).

The baseline concentration of MMP-9 in the tumour tissue extract samples was 89.9 ng/mL. After 12 years cryopreservation a significant increase (133.5 ng/mL) was seen ($p < 0.05$).

The Spearman rank correlation test was carried out on all pre- and post-cryopreservation samples. There was a distinct correlation between the samples of all sample types investigated. Spearmans rho as well as medians and ranges are reported in *Table 4*.

Table 4. Medians, interquartile ranges and Spearman rho pre- and post cryopreservation.

	BL analysis	P analysis	p value	ρ (p value)
MMP-9 P ng/mL (IQR)	9.9 (12.2)	9.7 (12.4)	0.13	0.96 (< 0.01)
MMP-9 HIT ng/mL (IQR)	7.1 (13.4)	8.1 (17.7)	< 0.01	0.97 (< 0.01)
MMP-9 TT ng/mL (IQR)	89.9 (175.4)	133.5 (234.5)	< 0.01	0.97 (< 0.01)

Abbreviations: BL = Baseline; P = Present; MMP = Matrix metalloproteinase; IQR = Interquartile range; P = Plasma; HIT = Healthy intestinal tissue; TT = Tumor tissue.

Bland-Altman plots (*Figures 10A-C*) were constructed to visualise the difference to mean of each analysis. In the plots the mean value of all pre-cryopreserved samples are indicated with a line and all values from each of the 36 samples are plotted as the mean of the pre- and post-cryopreserved paired samples (X axis) and the difference to the mean of all samples (Y axis). There is a greater spread from the mean axis in the tumour-tissue samples and the tumour-free intestinal tissue sample, compared to the plasma samples, where no significant difference was seen according to the Wilcoxon rank test.

The cryostability of MMP-9 measured in plasma and tissue extract was investigated in Study II. We used our own biobank and standardised sampling conditions to imitate a clinical, real-time set-up. MMP levels in plasma remained stable over a long period of time in cryopreservation, whilst tissue extracts from tumour and adjacent tumour-free intestinal tissue showed increases over time.

The Arrhenius equation is described in this paper since others have used it for calculation of MMP stability. Previous studies on real-time stability as well as calculations using the Arrhenius equation have shown MMP to be stable[117-119, 136], whilst *Rouy et al* found MMP-9 to dramatically decrease during storage[115]. Before our study, however, no one had studied real-time stability of MMP for such a long period of time. *Rouy et al* also used a different approach as they compared pooled samples of MMP that had been cryopreserved over different periods of time. The study in this thesis used paired samples from each patient.

In the study presented in this thesis, the ELISA technique was used to determine the level of MMP-9. MMP-9 is one of the most important enzymes involved in the degradation of ECM, and several studies have used MMP-9 when investigating biomarkers in CRC[102, 103].

The recovery of MMP from cryopreserved plasma samples was almost 100 % and there was no difference between baseline and final values according to the Wilcoxon rank test. Correlation was almost linear. However, an increase in MMP was seen in both types of cryopreserved tissue extract; the tumour extracts increasing the most. We cannot explain the reasons for this. There is a certain risk of cell damage and thereby MMP leakage during the tissue homogenisation process. Tumour tissue being more cellular could result in higher concentrations due to leakage compared to tumour-free intestinal tissue samples. There is also a risk that the paired aliquot taken from the extract contains more or fewer damaged cells. This cannot, however, fully explain why the frozen samples increase in MMP concentration. Further research is wanted to explain this phenomenon.
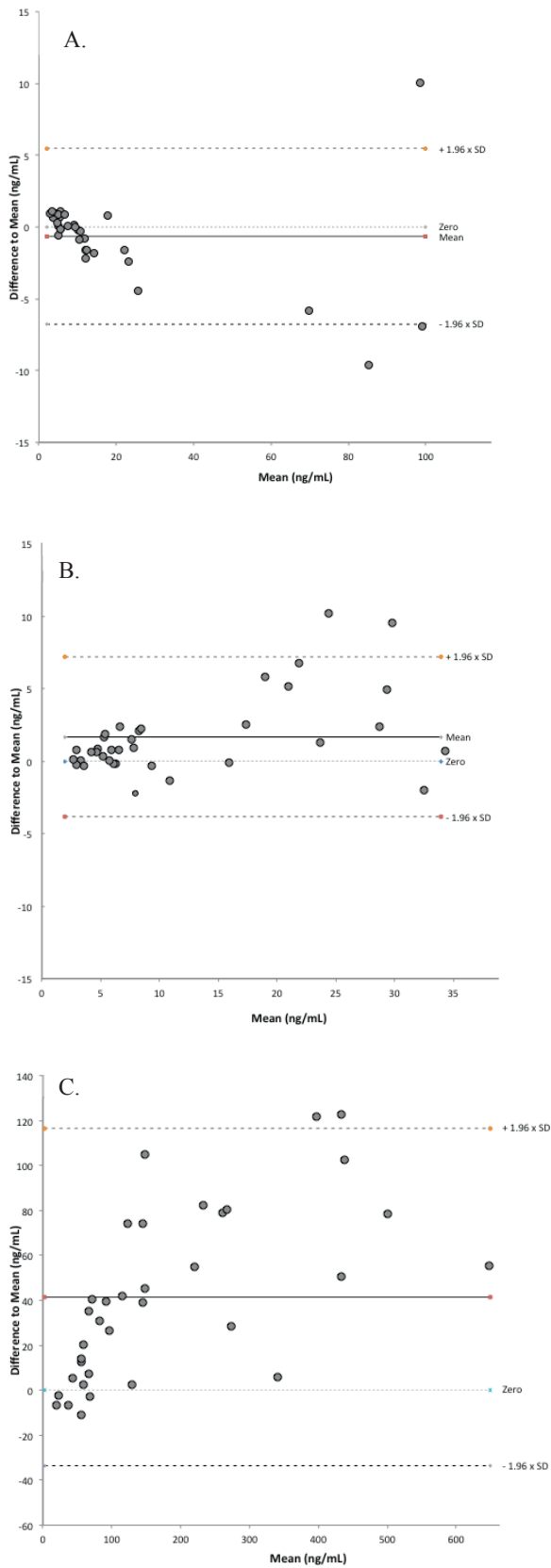
Figure 10A-C. Bland–Altman plot showing the difference to mean for each analysis. Dots that are between upper and lower dotted lines represent values within the 95% confidence interval. A; cryopreserved plasma samples, B; cryopreserved healthy tissue samples and C; cryopreserved tumour tissue samples.

In Paper II we used Spearman correlation analyses, and interestingly there were good correlations within all three groups investigated. This highlights the limitation of the correlation test, a strong linear relationship does not necessarily mean strong agreement between two tested concentrations before and after cryopreservation. The Bland-Altman plots reported in the paper visualise differences from the mean of each paired sample from the mean of the whole group. This gives a good picture of changes in concentration during cryopreservation, but it too has its limitations. The calculated 95 % confidence interval is a construct by the standard deviation of the analyses. As the CI is calculated from the mean difference they become somewhat misleading as the mean differs from the zero mark that represents no difference at all. This could, however, have been circumvented using a relative difference in the plots, but that postulates normally distributed data, which was not the case. The Bland-Altman plots should thus be seen as a visualisation tool rather than a statistical test[124].

In all, 36 samples of plasma and centrifugal extracts from tumour tissue and tumour-free intestinal tissue from patients operated for CRC were analysed. The baseline concentrations were known from previous research and samples were chosen to represent concentrations of MMP-9 in plasma as well as in tissue extract at both low and high levels. The samples were also chosen to have a relevant case mix at different stages of CRC. In some cases it was not possible to analyse the cryopreserved aliquot. To compensate for this the group was extended with further samples to achieve 36 paired samples in each group. The limitation of this is clear, as it did not keep to the definition of randomised sampling.

A second limitation of the present study is the use of different storage times. Due to the need to analyse samples in batches, storage times between sampling point and baseline analysis differed. Furthermore the time in storage between pre- and post-cryopreservation varied between plasma and tissue extract analysis.

We conclude that MMP levels in citrated plasma samples stored at -80 °C storage remain stable. This is important for retrospective as well as prospective research regarding plasma MMP as a biomarker in CRC. It also seems that MMP levels in tissue samples increase during long-term cryopreservation.

III. Plasma MMP Levels as a Prognostic Marker in CRC

The prognostic value of MMP levels in plasma was evaluated in Study III. A cohort of patients operated for CRC between 1999 and 2005 was used. Of a total of 331 patients, valid data were available for 272. Patients who received preoperative irradiation were excluded as irradiation affects MMP expression[137, 138].

Patient characteristics are shown in *Table 5*. Of a total of 200 patients included in the study, 48 patients were treated for rectal cancer and 152 patients were treated for colon cancer. Comparison was made with all patients that underwent planed surgery for colorectal cancer disease, under the same time period at the Sahlgrenska Hospital/Östra, Gothenburg, Sweden, in order to assess the degree of representation of our cohort, see *Table 5*. The median age of patients as well as cancer stage distribution was quite similar. There was a difference in the distribution of tumour localisation as the study cohort had relatively more patients with tumour in the sigmoid and fewer patients with right-sided colon cancer. There was equal division of cancers between the colon and rectum but there were more patients receiving neoadjuvant radiotherapy in the study cohort.

Using ROC curves the optimal cut-off value was estimated for each MMP. MMP-1 was found to have an area under the curve (AUC) significant larger than 0.5 and was therefore used in further analysis. The optimal cut-off value was a plasma concentration of 336 pg/ml and patients were divided between those with a *low* MMP-1 plasma value (i.e <336 pg/ml) and those with a *high* MMP-1 plasma value (>336 pg/ml).

Table 5. Patients characteristics, distribution of UICC stage, tumour localisation and the use of neoadjuvant radiotherapy and adjuvant chemotherapy.

	Study group	Total population of operated patients
Median age (IQR)	72 (65 - 80)	71 (62 - 79)
Males/Femals (%)	151/121 (55.9/44.1)	667/632 (51.3/48.7)
UICC Stage		
I (%)	41 (15.1)	188 (14.5)
II (%)	95 (34.9)	460 (35.5)
III (%)	99 (36.4)	477 (36.7)
IV (%)	37 (13.6)	163 (12.5)
N/A (%)		11 (0.9)
Total (n)	**272**	**1299**
Tumour localisation:		
Ascending colon (%)	66 (24.3)	410 (31.5)
Transverse colon (%)	8 (2.9)	27 (2.1)
Descending colon (%)	16 (5.9)	49 (3.8)
Sigmoid colon (%)	62 (22.8)	242 (18.6)
Total colon (%)	**152 (55.9)**	**728 (56.0)**
Rectum (%)	**120 (44.1)**	**571 (44.0)**
Adjuvant CMT		
Yes (%)	57 (21.0)	320 (24.6)
No (%)	215 (79.0)	979 (75.4)
Neoadjuvant RT, Rectal Cancer		
Yes (%)	72 (60.0)	253 (44.3)
No (%)	48 (40.0)	318 (55.7)

Abbrevations: IQR = Interquartile range; CMT = Chemotherapy; RT = Radiotherapy

Survival analyses were made using Kaplain-Meier curves. A *high* MMP-1 value was associated with poor cancer-specific survival in the total group of patients (p < 0.05). Analyses where then made regarding patients with non-disseminated colorectal cancer disease (*Figure 11A)* showing a significant difference in survival of patients with *high* and *low* plasma MMP-1 levels. In subgroup analysis of the Stage III group (*Figure 11B)* the Kaplain-Meier curve showed that a *high* plasma MMP-1 concentration was associated with poor cancer-specific 5-year survival. These differences in survival remained when 10-year cancer-specific survival was analysed.
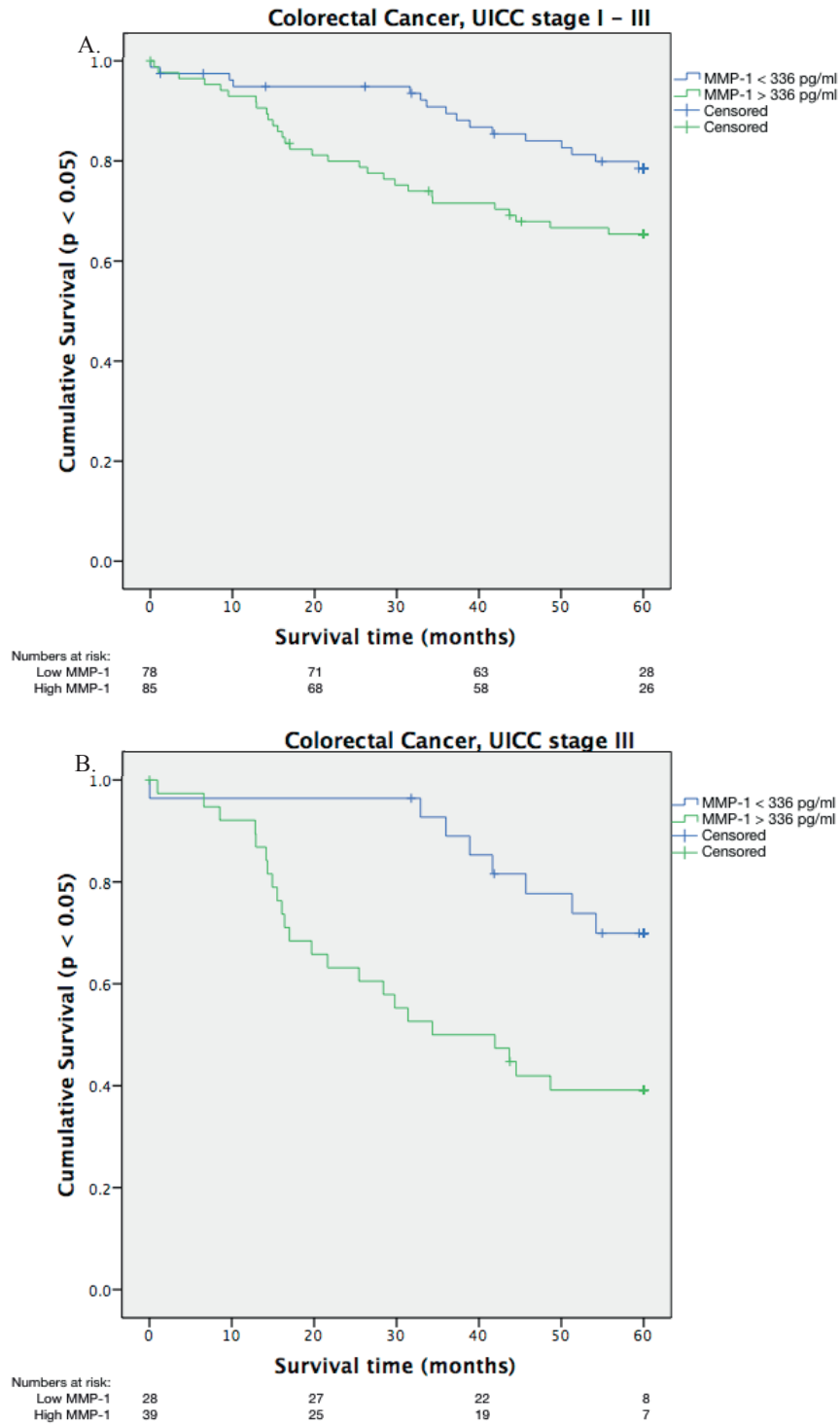
Figure 11A-B. *Cancer specific 5-year survival in patients with colorectal cancer, UICC stage I-III (A) and cancer specific 5-year survival in patients with stage III colorectal cancer (B). Divided in patients with high (>336 pg/ml) and low (<336 pg/ml) MMP-1 plasma concentration (p < 0.05)*

39

Using univariate Cox analysis, covariates were evaluated and used in multi-variable analysis for Hazard Ratio estimations. In patients with metastatic disease there was no association between MMP-1 level and survival, and neither age nor tumour localisation had an impact on survival. In patients with Stages I-III colorectal cancer, univariate analysis demonstrated that age, stage and a *high* MMP-1 plasma level were significant for 5-year cancer-specific survival. In multivariate analyses age and cancer stage were the only variables with a significant impact on survival. In analyses of overall 5-year survival in the Stages I-III cancer group, a *high* MMP-1 value, cancer stage and age all had a p-value below 0.16 in univariate analysis and were used in multivariate analyses where *high* MMP-1 level did not have an impact on survival

Patients with Stage III colorectal cancer (n = 67) were analysed in univariate analysis. Variables with a p-value below 0.16 were used in multivariate Cox analysis where a *high* level of MMP-1 in plasma was found to be a negative predictor of 5-year cancer-specific survival (31 events, HR 2.99, CI 1.15 - 5.87). In multivariate analysis of *overall* 5-year survival, only treatment with adjuvant chemotherapy was found to be of prognostic value. There were 54 patients with Stage III colon cancer. In this group a *high* MMP-1 value was a negative prognostic factor in multivariable analysis for 5-year cancer-specific survival (26 events, HR 2.7 CI 1.05 - 6.82).

The prognostic value of MMP-1 concentration in plasma was also evaluated by multivariate analysis for 10-year cancer-specific survival in patients with Stage III colorectal cancer (37 events, HR 2.91 CI 1.33 - 6.38). A *high* MMP-1 value was a negative prognostic factor of 10-year cancer-specific survival for patients with Stage III colon cancer (31 events, HR 2.55 CI 1.07 – 6.10).

In paper III the prognostic value of MMP measured in circulating plasma was evaluated and MMP-1 was found to have an impact of cancer specific survival. MMP concentrations in systemic blood have earlier been studied regarded colorectal cancer survival; this is shown in MMP-2, MMP-7, MMP-8 and MMP-9 where concentrations were measured in serum[101, 105, 106, 139, 140]. However, paper III studied levels of MMP in *plasma* samples that are known to be more stable than, serum[141].

The findings in paper III is of importance as our prognostic models for survival after colorectal cancer surgery are somewhat insufficient. Advanced stage II colorectal cancers as well as stage III are of special interest as this are the groups of patients receiving adjuvant chemotherapy follow surgery. Unfortunately, just

about 15 % of all patients receiving adjuvant chemotherapy avoid recurrence due to the chemotherapy treatment[44, 142, 143]. The prognostic value of MMP-1 in plasma for patients with Stages II-III colorectal cancer undergoing surgery could be of importance as a supplement to TNM staging when identifying patients for adjuvant treatment. As seen in *Figures 11A-B,* patients with a *high* MMP-1 value had a poor cancer-specific survival and it is shown that the largest difference in outcome for patients with *high* and *low* values are seen within the first three years. This is in concordance with previous findings where the majority of colorectal cancer recurrences have been reported to appear in the first three years after surgery, highlighting the need for improved prognostic models for survival in colorectal cancer disease[144]. In a further analysis (data not reported) we found that MMP-1 levels in plasma had a significant prognostic impact on survival in patients with Stage II – III colorectal cancer who did not receive adjuvant chemotherapy, see *Figure 12*. In multivariate analysis together with age and cancer stage a *high* MMP-1 level was a predictor of cancer-specific survival (35 events, HR 2.3 CI 1.09 – 5.01). These findings might be of importance regarding further improvement in the criteria required when deciding on adjuvant chemotherapy.
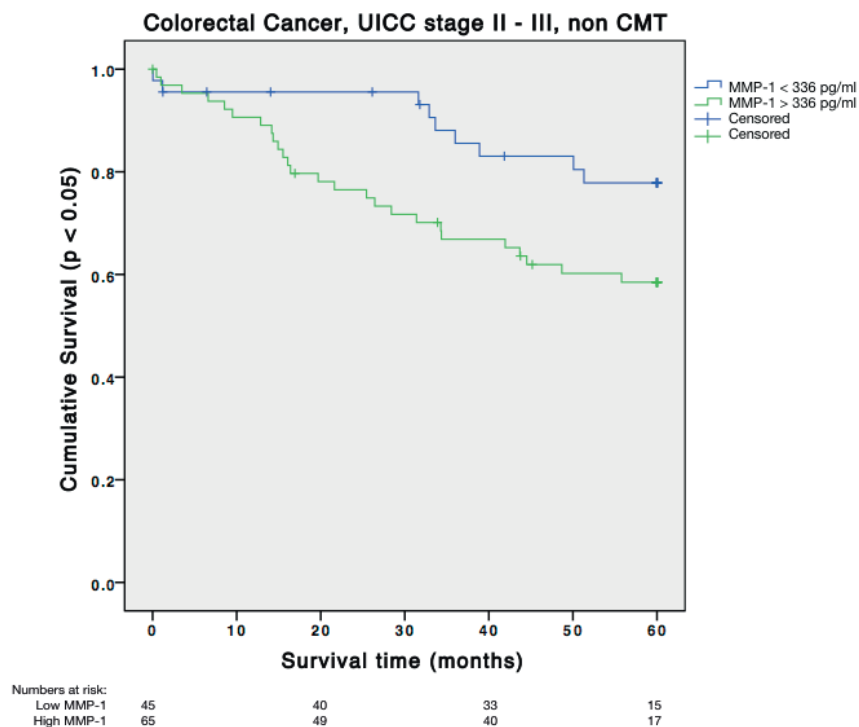

Figure 12. *Cancer specific 5-year survival in patients with colorectal cancer, UICC stage II-III without adjuvant chemotherapy. Divided in patients with high (>336 pg/ml) and low (<336 pg/ml) MMP-1 plasma concentration (p < 0.05)*

In paper III there were some limitations; the nature of long-term follow-up studies postulate that treatment protocol, surgical and pathological technique might have changed during the time period. There were relatively few patients with rectal cancer disease as patients receiving neoadjuvant radiotherapy were excluded.

We conclude that the findings reported in Paper III could support the use of MMP-1 plasma levels as a prognostic tool in patients treated for colorectal cancer. As well it might be of importance in the selection of patients that ought to receive adjuvant chemotherapy following colorectal cancer surgery. More studies are to be conducted to verify the results.

IV. The Use of *ex Vivo* and *in Vitro* Models to Study the Role of Surgical Trauma in Peritoneal Carcinomatosis

In paper IV a model to understanding the role of surgical trauma for cancer spread to the peritoneal surface was developed. The study was experimental.

The cell cultures of harvested mesothelial cells as well as Colo205 tumour cells were not affected by isolation, subcultivation or storage in liquid nitrogen. The *ex vivo* model of human peritoneum was kept without contamination for more than 14 days. Migrating fibroblasts were seen to be present indicating viability of the peritoneal cells after 8 days culture.

Following localised trauma to the mesothelial cell layer we observed that added Colo205 cancer cells adhered to the traumatised area to a greater extent than to the non-traumatised area. The same observation was made regardless of cell density of the Colo205 suspension added. Time-lapse photographs of adherence of tumour cells to the mesothelial layer are shown in *Figure 13*.
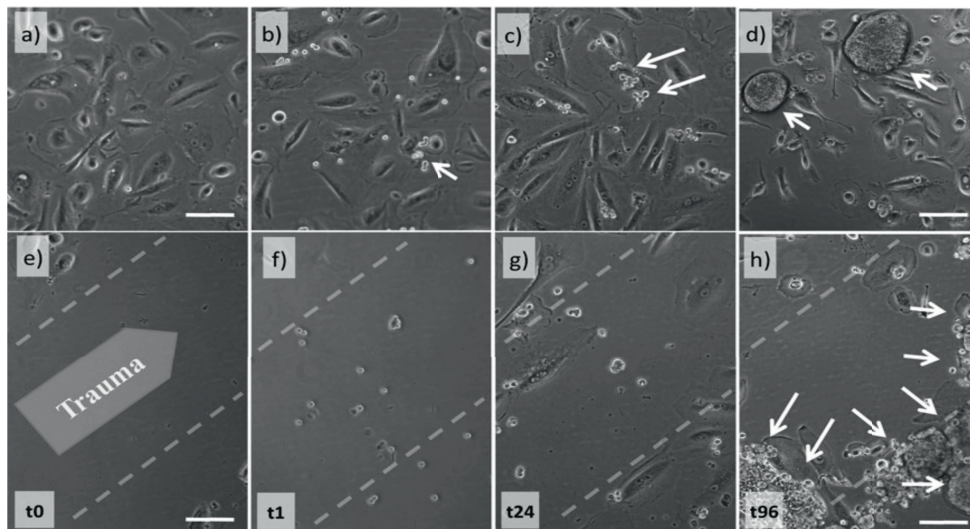

Figure 13. *Mesothelial cell layer trauma model with tumour cells added. Edges of the damaged area indicated by ----. A greater number of tumour cells were attached to the damaged mesothelial area (h) than to the undamaged (d), after 96 hours in culture.*

A tendency of tumour cells adhesion in clusters to the edge of the traumatized area was also seen in the peritoneal *ex vivo* model. As for the mesothelial cell model the same tendency was seen regardless of the cell density of the Colo205 suspension added, *Figures* 14a-f.
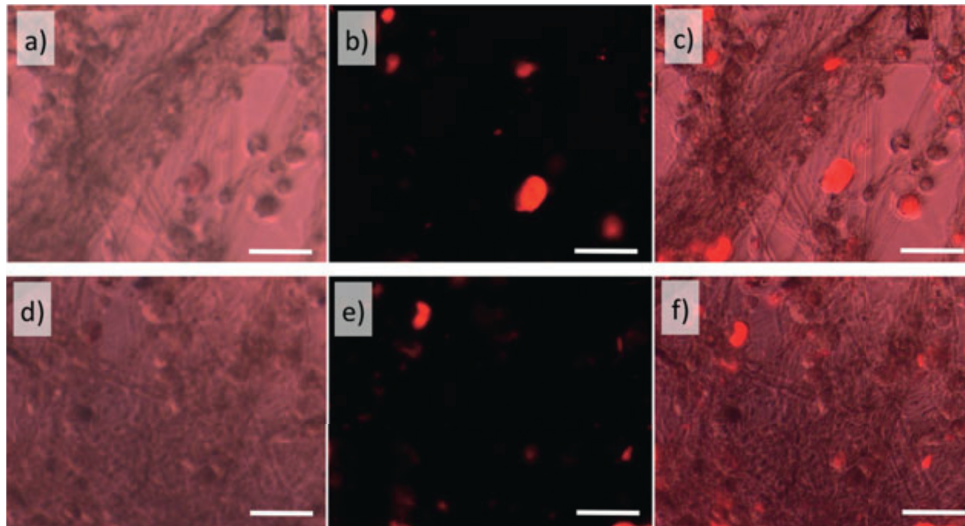

Figure 14. *Images of added tumour cells in the ex vivo human peritoneal membrane model. By combining the images from light microscopy of the peritoneal ex vivo model (a) with the labelled tumour cells (b), a summary image of labelled tumour cells could be seen on the peritoneal surface (c). Low cell density numbers (a-c) compared to high numbers (d-f). Tumour cells adhere to the edges in a similar manner to that seen with the monolayer of mesothelial cells.*

Paper IV may be seen as a form of validation of our constructed *ex vivo* and *in vitro* models. Due to the methods used in this experimental approach, no quantitative conclusions could be drawn.

This is not the first experimental model for studying the peritoneum *ex vivo*. Previous studies have been carried out in animal models using rats[145, 146] and mice[147, 148]. Research on human *ex vivo* peritoneal models has also been carried out, though the viability of that model was restricted to 72 hours compared to the model used in this thesis where viability was confirmed after 8 days[149].

In this paper we report that artificial surgical trauma appears to facilitate colon cancer cell adherence to the peritoneal surface. The reasons for this are not fully understood. Previous studies have suggested that surgical trauma induces mediators and proteases, including MMP, that facilitate adherence and metastasis to the peritoneal surface[97, 149, 150].

In conclusion, in our models mesothelial cell and peritoneal tissue were kept alive and could be under regular monitoring for a long period of time. Photo documentation revealed a tendency for cancer cells to adhere in clusters to the edges of the mimicked trauma.

Since the publication of this study, our group has proceeded by investigating the role of MMPs in peritoneal carcinomatosis originating from CRC. Using immune-histochemistry. Levels of MMP-1, MMP-2 and TIMP-1 were evaluated in tumour-free peritoneal tissue and tumour invaded peritoneal tissue from patients undergoing cytoreductive surgery. MMP-2 and TIMP-1 were mostly seen in tumour-invaded areas but were not found in tumour free peritoneum. On the other hand, MMP-1 was expressed in tumour-free areas and in the invasion zone, in proximity to the tumour, but not in the peritoneal areas invaded by tumour[95].

These models will be used to further investigate how colorectal cancer cells adhere and metastasise at the peritoneal surface They might also be useful in research investigating novel intra-abdominal cancer treatments.

Conclusions

- Levels of MMPs in systemic blood should be measured in plasma.

- Levels of MMPs in plasma remain stable even though the samples have been stored for a long time at -80°C.

- Levels of MMPs in cryopreserved homogenised tissue extract from tumour and tumour-free intestinal tissue are not as reliable as in plasma.

- MMP-1 levels in the plasma of patients undergoing colorectal cancer surgery could have prognostic value regarding cancer-specific survival

- The *ex vivo* human peritoneal model as well as the *in vitro* human mesothelial cell model may be used to study colorectal cancer cell invasion and intraperitoneal spread.

Future perspectives

This thesis focuses on colorectal cancer and the microenvironment around CRC tumours. The goal has been to validate the methodology of blood sample handling and of MMP measurement, in order to further investigate MMPs role as a prognostic marker in CRC. Our experimental models using peritoneal tissue and mesothelial cell culture were used to gain further knowledge in the methodology of *ex vivo* and *in vitro* studies for future research into the intraperitoneal spread of CRC and the role of MMPs in this process.

The results presented in this thesis imply that levels of MMP-1 in plasma, given that the samples investigated are handled and stored correctly, may have prognostic value regarding survival of patients with colorectal cancer. Accurate prognosis is of great importance in the selection of patients who are likely to benefit from adjuvant treatment following surgery. There are a large number of patients with CRC Stage II and Stage III disease where the TNM status system fails to help in the selection of patients likely to benefit from adjuvant treatment. The use of MMP-1 as a prognostic marker could play an important role in our efforts to improve selection of candidates for adjuvant treatment and to optimise follow-up programmes after colorectal cancer surgery. Postoperative follow-up is time-consuming and expensive and the benefit of intense follow-up program-mes on survival have been questioned[151].

There are several ongoing studies aimed to evaluate whether neoadjuvant treatment has a place in the treatment of patients with advanced Stage II and Stage III colon cancer. In our cohort of patients with colorectal cancer (data not reported), elevated levels of MMP-2 and MMP-9 were significantly higher in patients with Stage III disease. We have determined optimal cut-off levels for MMP-2 and MMP-9 providing sensitivity and specificity for lymph node involvement of 70.4 % and 61.1 % respectively. These figures are comparable with the accuracy of computed tomography in finding Stage III disease preoperatively, and MMPs may have a future role as biomarkers when stratifying patients with advanced colon cancer for neoadjuvant chemotherapy.

Our *ex vivo* model is now validated as the harvested peritoneal layer could be kept alive for long periods of time. The adhesion of Colo205 cells in clusters along the edges of trauma created in our model suggests that the surgical trauma itself might have an impact on intraperitoneal spread after CRC surgery. Our

model can be used as a tool in further investigation of the role of the surgical trauma in carcinomatosis. It can have other uses as well such as the study of the biology involved in tumour adhesion and invasion, following changes in various mediators and enzymes. Peritoneal tissue already invaded by peritoneal carcinomatosis could be used as a model to develop novel medical treatment for PC. HIPEC is gaining increasing interest as an alternative for patients with high risk for peritoneal recurrence due to colorectal cancer. Even here our model might have an important role in our efforts to understand the underlying biology of colorectal cancer spread to the peritoneal surface, especially since recent studies have reported difficulty in stratifying patients who stand to gain from prophylactic HIPEC treatment[152]. Tumour- associated macrophages (TAM) are thought to play an important role in colorectal cancer invasion, and it has been suggested that TAM can interact with cancer stem cells[153]. Our *ex vivo* model is a great tool to further investigate the role of TAM in colorectal cancer disease and our group are planning further studies in this field.

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Appendix

Paper I-IV