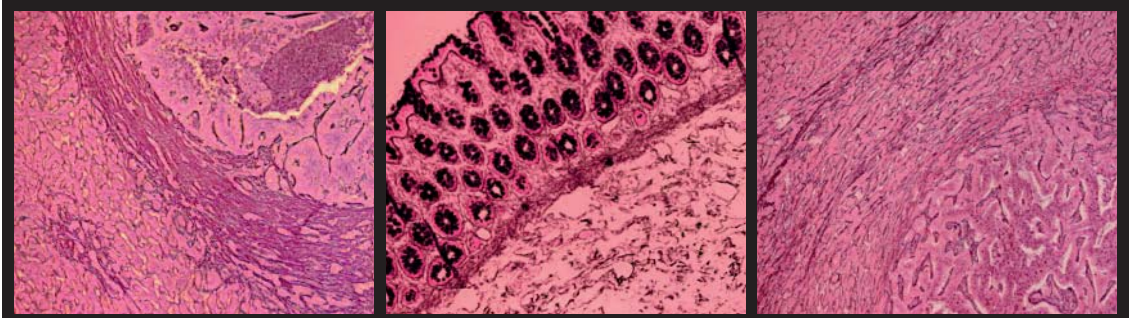

Stromal collagens in colorectal cancer and in colorectal liver metastases

Tumour biological implications and a source for novel tumour markers

Hanna Nyström

Department of Surgical and Perioperative Sciences, Section of Surgery
Umeå University 2013

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Abstract

Background: Colorectal cancer (CRC) remains one of the leading causes of cancer-related mortality. About 50 % of patients with CRC will develop subsequent liver metastases (CLM). The survival for untreated CLM is only a few months and liver resection provides the only chance for a lasting cure. It is therefore essential to detect CLM early, enabling successful surgical resection and achieving a long-term cure. There are no optimal tumour markers for CRC or CLM. The best marker available is Carcinoembryonic Antigen (CEA), a marker found elevated in about 50-60% of patients with CLM, but also in many other conditions.

The main focus of cancer research has been on the malignant cancer cell. However, a tumour consists of more than cancer cells. A major part of all solid tumours is made up by the stroma. The tumour stroma is defined as the non-malignant cells of a tumour such as fibroblasts, the cells of the vascular and immune systems as well as the extracellular matrix (ECM). The basement membrane (BM) is a specialized form of the ECM in which type IV collagen is the major protein component. All epithelial cells need a contact to the BM and the definition of an invasive cancer is the degradation of the BM and the spread of cancer cells beyond this structure. Different metastatic growth patterns of CLM have previously been described, namely the desmoplastic, pushing and replacement type of CLM. These differ in their stromal reaction in the border, which separates the tumour from the normal liver.

In this thesis the tumour stroma of CRC and CLM is studied with a special emphasis on stromal collagens. The aim is to investigate whether stromal collagens/ circulating type IV collagen can be used as tumour markers for CRC and CLM, and to compare this to the conventional marker CEA. The circulating type IV collagen level is also measured in liver metastases from other primary tumours than CRC. Furthermore, the differences between the stroma of a primary CRC that metastasizes to the liver when compared to a CRC that never spreads are analysed. Additionally, the metastatic growth pattern of CLM is studied in relation to the primary tumour, stromal components and survival. We also sought out to find whether CRC cell lines possess the trait to produce ECM proteins endogenously, and in response to a normal liver stroma in a novel organotypic model for CLM.

Methods: Expression patterns of type I, III and IV collagen were studied by immunofluorescence (IF), chemical staining and immunohistochemistry (IHC) in normal colorectal tissue, normal liver, CRC, CLM, benign liver lesions and in liver metastases of other origin than CRC. Circulating plasma levels of type IV collagen were analysed in healthy controls, patients with CRC (T stage I-III) and in patients with CLM. Samples were analysed at the time of diagnosis, during and after oncological and surgical treatment and at the time of relapsing or progressive disease. Additionally, circulating levels were analysed in patients with benign liver lesions and in liver metastases of other origin than CRC. The metastatic growth pattern of CLM was classified

according to earlier descriptions. CRC cell lines were studied regarding their production of type IV collagen. The growth, invasiveness and stromal production in CRC cell lines were also investigated in a new organotypic model for CLM using human liver specimens.

Results: Circulating type IV collagen levels are increased in patients with CLM and other epithelial-derived liver metastases, and is found normal in patients with primary CRC (stage I-III), with liver metastases from tumours of non-epithelial origin, benign liver lesions and in healthy controls. The type IV collagen levels in patients with CLM reflect the tumour burden in the liver, decreases in response to therapy and is found increased in progressive or relapsing disease. The combination of circulating type IV collagen and CEA increased the sensitivity and specificity for detecting CLM. Liver-metastatic CRC displayed an increased stromal production when compared to non-metastatic CRC, with an increased type IV collagen expression in the direct vicinity of the CRC cells. The earlier described growth patterns of CLM were verified, with the pushing type of CLM associated with a short survival and poor outcome. Furthermore, CRC cell lines possess the trait of endogenously producing type IV collagen. The novel organotypic liver model revealed that CRC cell lines grown in the context of normal liver stroma, devoid of other cells, does not elicit a desmoplastic reaction.

Conclusion: Circulating type IV collagen is a promising tumour marker for CLM, where the levels reflect the hepatic tumour burden and can detect disease relapse after liver surgery. The combination of the tumour markers CEA and type IV collagen is superior to CEA alone. The stromal composition of primary CRC predicts the risk of subsequent CLM and the metastatic growth pattern of CLM is related to survival.

Keywords: Colorectal cancer, colorectal liver metastases, tumour marker, stroma, type IV collagen, metastatic growth pattern

Abbreviations & Definitions

5-FU	5-fluorouracil [5-fluorouracil-1*H*-pyrimidine-2,4-dione]
α-SMA	α-smooth muscle actin
APC	Adenomatous polyposis coli
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
BM	Basement membrane
CAF	Cancer associated fibroblasts
CEA	Carcinoembryonic antigen
CIMP	The CpG island methylator phenotype
CLM	Colorectal liver metastases
CpG	Cytosine preceding guanine
CRC	Colorectal cancer
DAPI	4'6-diamidino-2-phenylindole (a DNA binding fluorophore)
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EP LM	Epithelial originated liver metastasis
ERCP	Endoscopic retrograde cholangio-pancreatography
FAP	Familial adenomatous polyposis
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FLOX	5-FU, leucovorine and oxaliplatin
FOLFIRI	5FU, leucovorine and irinotecan
HE	Haematoxylin and eosin stain
HSC	Hepatic stellate cells
IA	Intra-arterial
IP	Intra-peritoneal
LM	Liver metastases
MMP	Matrix metalloproteinase
MMR	Mismatch repair system
MSI	Microsatellite instability
MSS	Microsatellite stable
NC-domain	Non-collagenous domain
NE LM	Neuroendocrine originated liver metastasis
PBS	Phosphate buffered saline
RFA	Radiofrequency ablation
VEGF	Vascular endothelial growth factor

List of original papers

Paper I Nyström H, Naredi P, Hafström L and Sund M (2011) Type IV collagen as a tumour marker for colorectal liver metastases. *European Journal of Surgical Oncology* **37:** 611-617

Paper II Nyström H, Naredi P, Berglund A, Palmqvist R, Tavelin B and Sund M (2012) Liver-metastatic potential of colorectal cancer is related to the stromal composition of the primary tumour. *Anticancer research* **32**: 5183-5192

Paper III Nyström H, Tavelin B, Björklund M, Naredi P and Sund M (2013) Improved tumour marker sensitivity by combined type IV collagen and CEA measurements in colorectal liver metastases. *Manuscript submitted*

Paper IV Nyström H, Lundin C, Björklund M, Nyberg P, Salo T, Naredi P and Sund M (2013) Increased type IV collagen expression is a feature in liver metastases of epithelial origin and related to invasiveness of CRC cells in a novel organotypic liver metastatic model. *Manuscript*

Sammanfattning på svenska

Cancer i tjock- och ändtarmen (kolorektal cancer) drabbar över 6000 människor per år i Sverige och är en av världens vanligaste tumörsjukdomar. Hälften av patienterna med kolorektal cancer utvecklar dottersvulster i levern (kolorektala levermetastaser). Den enda hoppet för bot av levermetastasering är att dottersvulsterna kan opereras bort. Detta är möjligt för ca 15-20% av patienterna och ger en 5-års överlevnad på mellan 35-50%. Utan möjlighet till radikal kirurgi är överlevnaden endast ett par månader, men sjukdomen kan ibland bromsas ytterligare ett par månader med hjälp av cellgifter.

En biomarkör eller tumörmarkör är ett ämne som kan mätas i t.ex. blod eller urin och kan användas för att upptäcka en sjukdom, övervaka en behandlad tumörsjukdom för att upptäcka återfall, utvärdera given behandling t.ex. cellgifter och även användas för att bedöma sjukdomens allvarlighetsgrad och prognos. Det är dock svårt att finna sådana markörer då de skall vara helt unika för just den tumörformen och inte utsöndras vid annan sjukdom. Den vanligaste tumörmarkören för kolorektala levermetastaser är CEA (Carcinoembryonic Antigen), men bara omkring hälften av patienterna med levermetastaser har förhöjda nivåer av denna markör. Eftersom den enda möjligheten till bot är radikal kirurgi utav levermetastaserna är det avgörande att de upptäcks i tid. Det finns därför ett behov för nya och bättre tumörmarkörer.

En tumör består inte bara av elakartade (maligna) cancer celler, utan även av ett tumör stroma. Stromat består av bindvävsmolekyler och av icke-maligna celler såsom blodkärlens celler och immunförsvarets celler. Tumörstromat är viktig för cancerutveckling och metastasering. I stromat finns det ämnen som kan stimulera cancercellernas förmåga att dela sig, spridas och undvika celldöd. Bindvävsmolekylerna i stromat produceras sannolikt av både cancercellerna själva och genom att cancercellerna också påverkar stromats egna celler, så att i sin tur dessa producerar ett stroma som just gynnar cancercellerna. Ett basalmembran är en specialiserad del av bindväven, som epiteliala celler fäster sig vid i. Definitionen på en cancer är när detta basalmembran luckras upp och de elakartade cellerna sprider sig ner förbi basalmembranet ut i den övriga vävnaden i en process som kallas invasion.

I avhandlingen studeras olika sorters bindvävsproteiner i tarm tumören, nämligen typ IV kollagen som vanligtvis enbart finns i basalmembranen, och typ I och III kollagen som vanligtvis finns i vävnaderna under basalmembranen i tarmen. Vi studerar också dessa proteiners uttryck i levermetastaser, där typ III och IV kollagen vanligtvis finns i kärlens basalmembran, liksom i leverns finmaskiga nät som filtrerar blodet från tarmen och typ I kollagen som finns i leverns kärl och gallvägar. Vi tittar också på sättet som levermetastaserna växer på. Vi fann i kolorektala levermetastaser höga nivåer av framför allt typ I och IV kollagen. Vi jämförde också tarm tumören hos en grupp patienter som utvecklat levermetastaser med en grupp patienter som aldrig utvecklat spridd sjukdom och här såg vi att det i den lever-metastaserande tarmcancern fanns höga

uttryck av typ IV kollagen i nära anslutning till de invaderande cancercellerna. Vi fann också att levermetastaserna växer med olika mönster och att patienter med ett "pushing" typ av växtsätt hade mycket dålig överlevnad, trots kirurgi, medan patienter med ett "desmoplastiskt" växtmönster i hög grad botades från sin cancer efter leveroperationen.

Vidare mäter vi typ IV kollagen nivåer i blodet från patienter med kolorektal cancer och med kolorektala levermetastaser och jämför detta med hur mycket typ IV kollagen som finns i metastasvävnaden i levern. Detta för att se om typ IV kollagen kan användas som tumörmarkör för kolorektala levermetastaser och jämför detta med den mest använda markören CEA. Vi fann att typ IV kollagen i blodet är en mycket bra markör för att hitta levermetastaser och att patienter med kolorektal cancer har normala nivåer. Markören speglar också hur stora tumörerna i levern är och kan användas för att hitta återfall i sjukdomen efter leverkirurgi. När vi slog samman collagen IV och CEA i en gemensam analys visade det sig att denna kombination gav upphov till en mycket bra metod för att finna levermetastaser, ännu bättre än att använda endera markören separat.

Vi ville undersöka om kolorektal cancer (KRC) celler själva kan producera typ IV kollagen, därför lät vi sådana celler växa på plast. Där såg vi att några cellinjer kunde själva kunde producera typ IV kollagen, medan andra cellinjer inte har den förmågan.

Vi ville också utveckla en ny experimentell modell för att studera processen kring levermetastasering i laboratoriemiljö, utan att behöva använda försöksdjur, men med en mer äkta vävnadsmiljö. Därför använde vi normal human levervävnad som opererats bort pga. cancer. Denna levervävnad var fryst och innehöll inga levande celler, utan bara det intakta bindvävsstromat. Därmed kunde vi undersöka om KRC celler kunde invadera denna lever och det kunde bara de cellinjer som producerade eget typ IV kollagen. Vidare undrade vi om det intakta leverstromat och KRC cellerna själva kunde orsaka en sådan bindvävsreaktion som man ser i riktiga levermetastaser. Det visade sig att någon sådan reaktion ej kunde ske, och att det alltså behövs fler komponenter, såsom stromala celler för att orsaka en sådan reaktion.

Sammanfattningsvis visar denna avhandling att basalmembrans-proteinet typ IV kollagen har potential att användas som tumörmarkör i blod för kolorektala levermetastaser och ger en ökad chans att hitta dessa, I synnerhet om man kombinerar den med tumörmarkören CEA. Dessutom verkar typ IV kollagen ha betydelse för den kolorektala cancerns förmåga att sprida sig till levern och att cancer celler själva tycks kunna producera detta. Vi har också hittat olika växtmönster av kolorektala levermetastaser som spelar stor roll för patientens chanser till överlevnad. Avslutningsvis har vi utvecklat en ny modell baserad på human levervävnad för att kunna studera processen kring levermetastaser med tyngdpunkt på den stromat. En tumör består av mycket mer än bara cancer celler och stromats betydelse i kolorektal cancer och vid kolorektala levermetastaser är av viktig betydelse, både som en källa till nya tumörmarkörer men också för tumörbiologin vid denna sjukdom.

I. Introduction

Colorectal cancer (CRC) remains as one of the leading cancer forms worldwide. Half of the patients will develop colorectal liver metastases (CLM) and the only chance to lasting cure is surgical resection of the tumour. Better understanding of tumour biology has improved the survival of CRC and CLM patients, but much is yet to be done in this field. The tumour stroma has emerged as an area of interest, since a tumour is highly dependent on its stromal context and microenvironment. This is true in both primary and metastatic cancers. To understand which processes that drive tumorigenesis, research should not only focus on the primary tumour, but also on the metastatic part of the disease. This however presents a tremendous challenge, since much less research material is available from metastatic patients, when compared with to patients with a primary tumour.

In this thesis the focus is both on the primary tumour and also on the metastatic part of the disease. The aim of this thesis is to study the stroma and especially the extracellular matrix (ECM) compartment in colorectal cancer (CRC) and in colorectal cancer liver metastases (CLM). The introduction will summarize the background for the project with a special emphasis on the surgical and oncological perspectives of CRC, and especially CLM. The thesis has a focus on the tumour stroma and also characterization of new tumour biomarkers for CLM. Thus these topics will be introduced in detail.

Chapter 1: The Colon and the Rectum

The colon and rectum is the last part of the digestive system, with a total absorptive area of about 1000 cm^2. The colon not only absorbs fluid and electrolytes from the bowel contents, but also hosts a complex bacterial flora where this symbiosis provides the body with essential substances such as Vitamin K. The knowledge of macro- and microanatomy, as well as function of the colon and rectum is the basis of successful oncological surgery in colorectal cancer (CRC).

1.1 Anatomy

The colon and rectum is about 150 cm in length, with the proximal end beginning at the ileocecal valve, where the terminal ileum empties its contents into the first part of the colon, the *cecum*. The length of *cecum* is about 10 cm. The *appendix* originates from the end of the cecal pole, at the convergence of the *taenia coli*. *Colon ascendens*, approximately 15 cm in length, follows after *cecum* and stretches upward towards the liver. Here the posterior colon is fixated against the retroperitoneum.

Colon transversum then follows after the fixed point at the hepatic flexure, being about 45 cm in length. The anchor point of the hepatic flexure is created by the nephrocolic ligament and lies over the right kidney, duodenum and porta hepatis. The transverse colon ends at the second fixed

point of the splenic flexure. The transverse part is enclosed in the visceral peritoneum. The fixed point at the splenic flexure is composed by the phrenocolic ligament and anchors this part of the colon to the left upper quadrant. This point is placed higher and deeper compared to where the hepatic flexure anchors, making this angle of the intestine in general much sharper. *Omentum Majus*, a double fused layer of *peritoneum visceralis* and *parietalis* originates from the superior part of colon transversum. After the splenic flexure *colon descendens* originates with a length of about 25 cm.

At the level of the pelvic brim *colon descendens* transcends into *colon sigmoideum*. The sigmoid part of colon has a thicker wall than *colon descendens* and is more mobile. The length varies between individuals being as short as 15 and as long as 50 cm. Colon sigmoideum has a long mesentery and often forms a loop in the pelvis.

At the level of the sacral promontory, colon sigmoideum transcends into *rectum*. Approximately at this point the taenia coli converges. The rectum is about 12-15 cm in length and lacks teniae coli and appendices epiploica. The sigmoid part and the rectum act as a fecal reservoir. Rectum lies close to the sacral curvature of the pelvis and its posterior part lies extra peritoneal, adhering this part to the presacral soft tissues. Posterior the rectum is the mesorectum, with a clinically important structure in surgical oncology, namely the rectosacral fascia/ fascia propria. The distal end of rectum is where the anal canal begins. The gross anatomy of the colon and rectum is visualized in **Figure 1.**

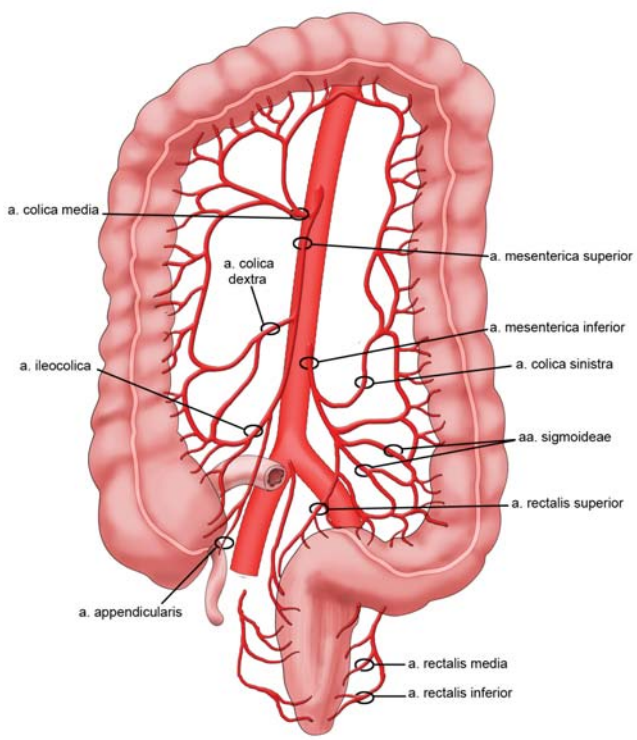

Figure 1. *Illustration of the anatomy of the colorectum and the arterial supply.* *© G. Andersson 2013*

1.1.1 Vasculature and lymphatic drainage

Arteria mesenterica superior and its three main branches *a. ileocolica, a. colica dextra* and *a. colica media* provides *cecum, colon ascendens* and part of *colon transversum* with blood. *A. mesenterica inferior* with its main branches *a. colica sinistra, aa. sigmoidales* and *a. rectalis superior* supplies the left part of the colon from the splenic flexure, down through *colon descendens, colon sigmoideum* and *rectum*. The anal canal also is supported by blood from *a. rectalis medialis*, which originates from a branch of *a. iliaca interna* and also from *a. rectalis inferior*, originating from *a. pudendus interna*. However, there are many collateral vessels providing arterial blood supply to the colon and rectum, such as *a. marginalis*, providing collateral flow between *a. mestenterica superior* and *inferior* as well as individual anatomic variations.

The venous drainage of the colon and rectum mirrors the arterial flow. Venous blood from *cecum, colon ascendens* and *transversum* empties into *v. mesenterica superior,* which fuses with *v. splenica* to become *v. porta*. Similarly *colon descendens, sigmoideum* and *rectum* drain into *v. mesenterica inferior*, which drains into *v. splenica*. The venous blood of the anal canal drains through the *vv. rectalis* directly into the *v. iliaca interna* and which then transcends into *v. cava inferior*.

Similarly, the lymphatic drainage follows the blood supply from the colon and rectum, which are supplied with a rich network of lymphatic capillaries. Lymph nodes are situated epicolic along the bowel wall and the epiploicae. Paracolic nodes are located adjacent to the marginal artery; intermediate nodes are found nearby the larger vessels and the primary nodes at *a. mesenterica superior* and *inferior*. The lymph from colon and the uppermost 2/3 part of rectum is drained into *nn. Paraaortales*, which then empties into *cisterna chyli*. Lymph from the lower rectum and anal canal is drained into *nn. paraaortales* or laterally by the lymphatic system of *iliaca interna*. A thorough understanding that vasculature and lymphatic's follow the embryonic planes is a keystone in current oncological surgery of the colon and rectum.

1.2 Microanatomy and function

The basis of the histology of the colon and rectum is illustrated in **Figure 2**. The most luminal layer is the *mucosa*, which is thinner in the cecum and thickest in the rectum. The mucosal layer consists of the crypts of Liberkuhn that are oriented as straight tubular glands to the crypt bases. The cells in the mucosa are mainly absorptive enterocytes presented as single columnar epithelium, but also mucus producing Goblet cells. Thus the main function of the colon is to absorb fluid and electrolytes from the bowel contents and to provide mucus, which facilitates the passage of content. A part of the mucosa is the *lamina propria* that contains the basement membrane (BM) of the colonic cells and is rich in lymphoid cells and tissue. Under the mucosa lies the *submucosa* that is composed of mainly loose connective tissue. The next layer is the *muscularis propria* or *muscularis externa,* which is created by

the muscular wall of the colon, where the inner layer is composed of circular muscle fibers and the outer layer by longitudinally oriented fibers organized in three bands, namely the *teniae coli*. This muscular organization makes it possible for parts of the colon to contract independently from one another, transporting the stools. The main part of the colon is covered by the *serosa*, whereas parts of the ascending and descending colon are covered by *adventitia*. The understanding of basic histology of the colon and rectum is important for the staging of colorectal cancer, further discussed in Chapter 3.

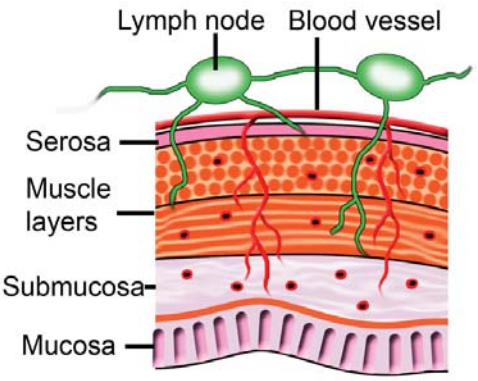

Figure 2.
The histological structure of the colorectum.
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Chapter 2: The Liver

The liver weighs about 1.6 kg in the adult and has over 400 known functions such as protein synthesis, detoxification and plays a key-role in metabolism. Despite much research, no medical intervention except liver transplantation can cure or compensate a failing liver. There has been many different ways to classify both the macroanatomy as well as the microanatomy of the liver, underlining its highly complex structure and function. Knowledge of the functional anatomy of the liver is the base upon which modern liver surgery rests.

2.1 Anatomy

The liver is almost completely protected by the rib cage, with only the lower part protruding below the costal margin. The superior surface of the liver is directly in contact with the diaphragm. The posterior surface straddles *v. cava inferior*. A wedge of the liver passes to the left of the abdomen, and lies anterior directly above the stomach and under the central and left part of the diaphragm.

The liver is enveloped in the peritoneum, except at the bed of the gallbladder and *porta hepatis*, and to the posterior on each side of the *v.*

cava inferior. The peritoneal duplications create the ligaments of the liver. *Ligamentum coronarium* is composed by the diaphragmatic duplication. *Lig. falciforme* emerges from the center of *lig. coronarium* and connects the surface of the liver to the diaphragm, abdominal wall and the umbilicus. *Lig. teres* forms from the lower part of *lig. falciforme* from the umbilicus to the umbilical fissure, which contains the left portal triad. *Lig. falciforme* was earlier used to mark the division of the right and left lobe of the liver, but is inaccurate and not useful for this purpose. On the posterior part of the liver is *lig. venosum* where *v. porta hepatis sinister* runs to *v. hepatica sinister* to empty into *v. cava inferior*. The venous blood carrying nutrients from the gastrointestinal tract drains into *v. porta hepatis*, which enters with the arterial blood of the liver in *a. hepatica*. These two large vessels enter into the hilum of the liver with the bile ducts, creating the portal triad. All venous blood of the liver is emptied into the *v. cava inferior*.

2.1.1 Functional anatomy of the liver

As mentioned earlier, historically the liver was divided into the right and left lobe by *lig. falciforme*. This classification in anatomically incorrect, since it does not reflect the vascular supply of the liver.

The liver is now divided into the left and right lobe determined by the left and right portal and hepatic vein branches. The liver is composed by eight segments (**Figure 3**), where each segment is supplied by a single portal triad (portal vein, hepatic artery and bile duct). These eight segments are then further organized into four sectors, separated by scissurae containing the three main hepatic veins. These four sectors are then even further classified into the left and the right liver lobe. This anatomical classification was pioneered by studies of Hjortsjö [1] and then extended and developed by Woodsmith and Goldburne in 1957 as well as by Couinaud [2] and is based on the structural organisation of the liver, which is today's platform for surgery of the liver. It however cannot be visualized on the surface of the liver.

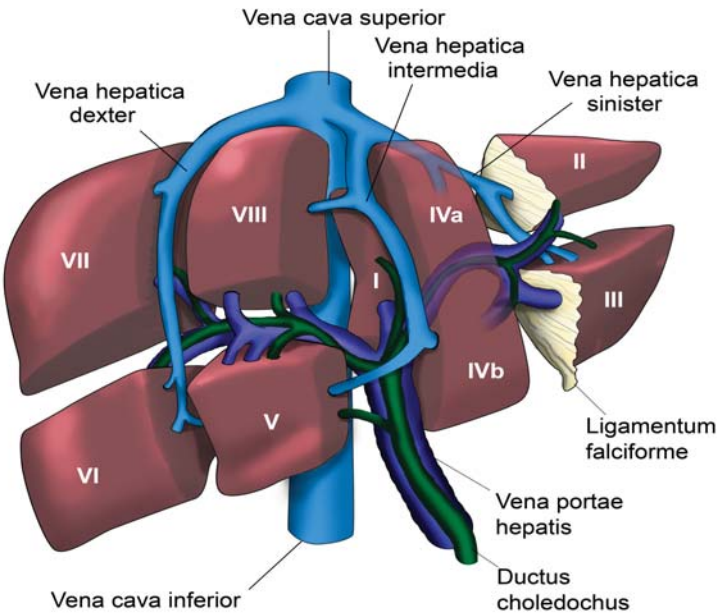

***Figure 3**. The segmental anatomy of the liver. © G. Andersson 2013*

2.2 Microanatomy and function

There have been many proposals on how the microanatomy of the liver should be visualized. Simplified, the different classifications reflect the different anatomic, metabolic and physiologic functions of the liver.

In this chapter I will focus on the *lobular* or the *acinar* classification of microanatomy. This classification was first presented by Rappaport [3] and later modified by Matsumoto and Kawakami.

2.2.1 The hepatic lobule

A lobule is composed by a central terminal hepatic venule surrounded by 4-6 terminal portal triads. This forms a polygonal unit (**Figure 4**). Between the peripheral portal triads hepatocytes are arranged in a single cell layer onto the endothelial-lined sinusoids filled with blood. This endothelial layer is highly fenestrated enabling passage of substances. The blood (both arterial blood from a. hepatica and venous blood from v. portae) then flows from the terminal portal triad through the sinusoids and empties into the central hepatic venule. The hepatocytes filter the blood, produce bile and excrete the bile into the terminal canaliculi, which then forms into larger bile ducts emptying bile into the bile ducts of the terminal portal triads.

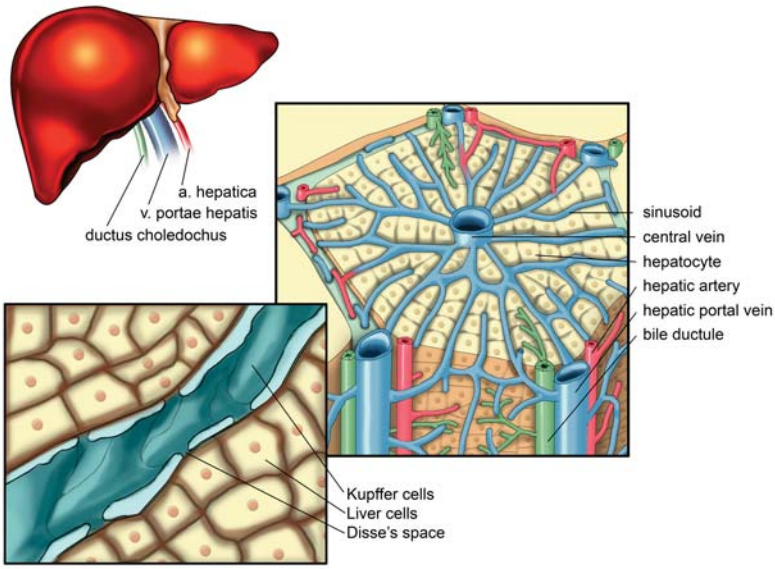

Figure 4. *Illustration of the hepatic lobule and the space of Disse. © G. Andersson 2013*

2.2.2 The Hepatocyte

Hepatocytes are highly complex cells with many functions and make up 60% of the cellular mass in the liver. The hepatocyte shape is polyhedral and the nucleus is centrally positioned. The hepatocyte is rich in organelles since it is one of the most metabolically active cells in the body. Each hepatocyte has contact with neighbouring hepatocytes, the bile canaliculus, and the sinusoidal space [4] [5]. Simplified the hepatocytes functions are uptake, storage, release of nutrients; synthesis of various components such as plasma proteins, glucose, fatty acids and lipids; production and secretion of bile as well as degradation and detoxification of toxins. The *basolateral* surface of the hepatocyte is exposed to the sinusoids and contains microvilli protruding through the fenestrae of the endothelial cells into the Space of Disse/perisinusoidal space. This surface provides active transport of substances from the sinusoidal blood. The *lateral* surface of the cell contains gap junctions, which allow for intracellular signalling, making communication possible between hepatocytes. The first part of the bile system, the bile cancaliculi/ the *canalicular* surface is formed by two opposing hepatocytes and is structurally secured by tight junctions between the cells, which contributes to maintain cell polarity [6] as well as intermediate junctions, gap junctions and desmosomes. These structural junctions create a barrier for larger molecules. The orientation of hepatocyte and its relation to the space of Disse is visualised in **Figure 4**.

2.2.3 The Hepatic Stellate cell and Kupffer cell

Hepatic stellate cells (HSC) are present in the Space of Disse (Chapter 2.2.4). These cells somewhat resemble pericytes, cells that are normally found on the outside of endothelial cells and the vascular basement membrane in vessels. HCSs are thought to be of mesenchymal origin and have variety of functions such as ECM production in both the normal and the fibrotic liver [7,8], a function to control of the microvasculature tone in the liver in a pericyte-like manner, to store vitamin A, and these cells play a significant role in the process of liver regeneration in both the normal and the injured liver [7,9]. HSCs also possess the potential to differentiate into other cell types, such as α-SMA (Smooth muscle actin) positive myofibroblasts, involved in diseases such as liver fibrosis. This differentiation is characterized by HSCs loosing their retinyl-ester stores, and differentiation into an active myofibroblast state and are immunohistochemically positive for α-SMA. The process is initiated by inflammation via the release of cytokines through the Kuppfer cells, but HSC contribute to the inflammatory response themselves by acting as antigen-presenting cells.

Kupffer cells are hepatic macrophages and are found in the lumen of the sinusoids. These cells are a specialised form of macrophages and are of significant importance in host defence mechanisms, clearance of endotoxins from portal blood and additionally possess migratory capabilities. They also are involved in many diseases of the liver.

2.2.4 The Space of Disse

The space of Disse, also known as the perisinusoidal space, lies between the sinusoidal wall and the surface of the hepatocyte facing the sinusoid. The hepatocyte has microvilli protruding into the space of Disse. The endothelial cells of the sinusoid do not have a normal BM. Instead of a conventional BM there is a reticular network composed of different ECM proteins and the composition of the ECM proteins varies in a portal gradient manner. The absence of a normal BM and the fenestration of the endothelial cells facilitate the two-way exchange between the blood and the hepatocytes. Hepatocytes express two integrin receptors (α1β1 integrin, a receptor for collagens and laminin, and also α9β1 integrin) which anchors to the ECM components in the basolateral surface of the cell [10]. The stromal composition of the liver will be further discussed in Chapter 6.2.

Chapter 3. Colorectal cancer

3.1 Incidence

Colorectal cancer (CRC) is the third most common malignancy worldwide. Each year 1.2 million people are diagnosed [11]. The overall mortality in CRC patients exceeds 50 % [12]. The highest incidence is found in countries with a western life-style thus identifying risk factors such as dietary habits, obesity

and smoking, whereas the lowest incidence is found in developing countries [11,13]. Men and women are almost equally affected by this disease [11]. In Sweden in the year 2010, the incidence for colon and rectal cancer was 4100 and 2103 patients, respectively [14]. The age-standardized mortality of CRC in Sweden has decreased since 1980 and survival has greatly improved [15], with a reported five year survival of 65% for women and 62% for men in colon cancer, and 64% for women and 62% for men in rectal cancer [14].

3.2 Etiology

CRC is influenced by both genetic and environmental factors, such as most cancers. Age, like in the majority of cancers, is the single most important risk factor [15]. There are also known inherited mutations responsible for familiar CRC cancers such as Familial Adenomatous Polyposis and Lynch Syndrome, but these only count for a small number of CRC patients.

Risk factors for developing of CRC are smoking [16,17], alcohol [17], inflammatory bowel disease [18] and in general a Western life style and diet. As an example, there is evidence that a diet high in red and processed meat increases the risk of CRC [19]. Doll & Peto have shown that up to 90% of the variations in CRC incidence between countries can be explained by dietary habits. Evidence supporting this is that the incidence of CRC in developing countries rises when the country adopts a western lifestyle. Additionally, when immigrants from low incidence countries settle in western countries, the incidence of CRC in this population will increase in a few generations [12,19].

3.3 Pathogenesis

The majority of CRCs are thought to arise from premalignant adenomatous polyps. This is referred to as the adenoma-carcinoma sequence. During this process a local lesion in the mucosa forms an initially benign mass that through subsequent epigenetic and genetic changes eventually becomes malignant (**Figure 5**).

The mutation of APC (Adenomatous Polyposis Coli), a tumour suppressor gene is considered to be a keystone event, and is found in both premalignant adenomas, as well as in CRC [20]. The importance of the APC gene is further underlined by its role in the majority of patients with FAP, where this mutation causes massive amounts of polyps in the colon and rectum, which will become malignant and develop into CRC.

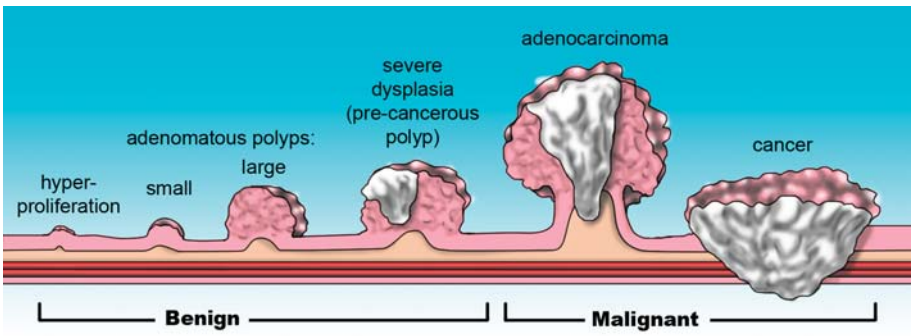

Figure 5. *The adenoma-carcinoma sequence where a benign polyp malignifies and establishes an invasive cancer. © G. Andersson 2013*

However, a cell needs to accumulate several mutations in order to become malignant [21]. A normal cell and organism however have extensive repair systems to prevent DNA damage. There are underlying reasons why malignant cells present with a number of mutations and different pathways of CRC tumorigenesis have been described: the microsatellite instable (MSI) pathway, the microsatellite stable (MSS) pathway and the CpG Island Methylator Phenotype (CIMP).

Microsatellites are repetitive sequences of DNA that are vulnerable to malfunction of the DNA replication machinery. The MSI pathway is driven by mutations in the mismatch repair genes (MMR), where the microsatellites of DNA become unstable and can be either shorter or longer. These abnormal repetitive DNA sequences are a sign of a faulty DNA repair system. The MSI pathway makes up for about 15% of the CRC patients. Common known mutations in MSI tumours are in found the genes for β-cathenin, BRAF. BAX, TGFβRII and IGFIIR [22]. A familial form of MSI is HNPCC or Lynch syndrome, which is caused by germline mutations in the mismatch repair genes (MLH1, PMS2, MSH6, MSF2)[23].

The MSS pathway is the major cause of CRC and displays chromosomal instability. A number of events have been found in MSS such as mutations in the APC, TP53, K-RAS and CTNNB1 genes [24]. The cause of this chromosomal instability can be due by several malfunctions such as abnormalities in telomere function, DNA damage response, mitotic checkpoint and the numbers of centrosomes.

The CIMP pathway is characterized by hypermethylation of promoter regions and was first described in 1999 [25]. These promoter regions consist of cytosines preceding guanines (CpGs) clustered in islands. They can also be found as single nucleotides in the genome. When these islands are methylated gene function will be silenced. In a subgroup of CRC these CpG islands are found hypermethylated thereby silencing important tumour suppressor genes. Tumours are referred to CIMP-negative, CIMP-low, CIMP-intermediate and CIMP-high, depending on the degree of hypermethylation.

These three pathways of tumorigenesis in CRC present different features, but also coexist, and overlap with each other thus mirroring the heterogeneity of cancer.

3.4 Symptoms and diagnosis

In early stages of the disease, there are no symptoms. Tumours located in the left part of the colon more often present with a change of fecal habits, bloody or mucus-deposited stool. Rectal cancer can present with urgency to empty the bowel, as well as blood and mucus in the stool. Right-sided cancers more rarely give these symptoms, but can give rise to more general malignant symptoms such as anemia, fatigue and weight-loss. If the tumour is obstructing the bowel, patients can present with pain, ileus and perforation of the bowel wall.

The golden-standard for diagnosis is colonoscopy or rectoscopy with the possibility to biopsy the lesion for histological verification of the diagnosis. After the diagnosis the patients are subject to more extensive clinical and radiological examinations to find signs of metastatic or locally advanced disease. This is essential to obtain information for a correct staging of the disease, thus making the best treatment for each individual patient possible.

3.5 Staging and survival

The staging of CRC was for many years preformed according to the Duke´s classification. This was later replaced by the TNM system that in general resembles the Duke´s classification. This system was developed and modified by UICC (Union Internationale Contre le Cancer) in 1987 and is today applied to most cancer forms. The TNM system stages patients in stage I-IV. Overview of the TNM staging of CRC and survival related to stage is found in **Figure 6** and **Table 1**.

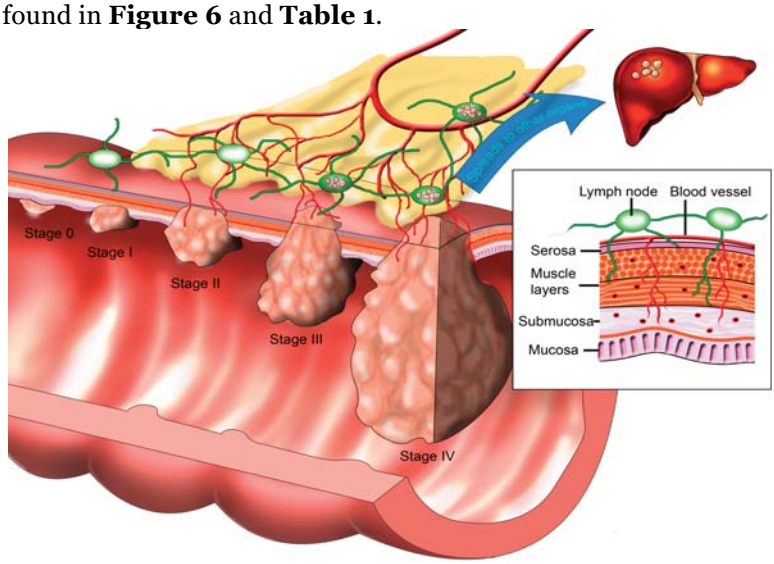

***Figure 6**. Illustration of the principles of TNM staging in colorectal cancer, based on tumour stage (T), nodal involvement (N) and presence of metastases (M). © G. Andersson 2013*

Table 1. *The relation between TNM and the Duke´s staging systems and survival*

TNM stage		**Duke´s stage**	
Tx	*Primary tumour can not be assessed*		
To	*No evidence of primary tumour*		
Tis	*Carcinoma in situ*		
T1	*Tumour invades the submucosa*	A	*Tumour has not penetrated beyond muscularis propria*
T2	*Invasion, but no penetration of muscularis propria*		
T3	*Penetration through muscularis propria and into subserosa or pericolic/rectal fat. but not to visceral peritoneum or other organs*	B	*Tumour has penetrated beyond muscularis propria, no lymph node involvement*
T4	*Invasion of other organs or the visceral peritoneum*		
No	*No nodal involvement*		
N1	*1-3 pericolic/rectal nodes involved*	C	*Lymph nodes involved*
N2	*>3 pericolic/rectal nodes involved*		
N3	*Any lymph node along named vascular trunk and/or metastasis to apical node(s)*		
Mx	*Presence of distant metastases unknown*		
Mo	*No distant metastases*		
M1	*Distant metastases*	D	*Distant metastases or local spread to adjacent organs*

TNM grouping				**Duke´s**	**5-years survival**
Stage 0	Tis	No	Mo	-	>95%
Stage I	T1	No	Mo	A	85-95%
	T2	No	Mo		
Stage II	T3	No	Mo	B	50-80%
	T4	No	Mo		
Stage III	Any T	N1	Mo	C	30-50%
		N2	Mo		
		N3	Mo		
Stage IV	Any T	Any N	M1	D	<5%

3.6 Surgical treatment

Surgery is the only way CRC can be cured, as is true for most solid cancers. Additional benefits for survival come from chemotherapy, radiotherapy and biological agents. But if the patient is not resectable, all other treatment will only have a palliative effect. The approach of surgical oncology and oncological medicine differs depending mainly on tumour localisation and TNM stage.

3.6.1 Rectal cancer

The surgical treatment of rectal cancer has drastically changed over the past decades thereby improving the survival for rectal cancer patients. Previously rectal cancer patients had a significantly poorer outcome than patients with colon cancer, but that has now changed with some Nordic countries reporting a better survival for rectal cancer patients than for patients with colon cancer [26,27]. There are different surgical approaches, depending on tumour localisation, and whether the patient is appropriate for surgical solutions that enable continency thus avoiding a stoma. However, the surgical technique used is to follow the embryonic planes, thereby dissecting in avascular planes and maintaining tissue plane integrity. This means that an intact surgical specimen is created with all the blood vessels, lymph vessels and lymph nodes intact, through which the cancer usually spreads. For the middle and the lower part of the rectum the golden-standard is total mesorectal excision (TME). TME was presented in 1986 by Heald who presented less local recurrence of CRC in these patients when compared to conventional surgery [28]. In TME the rectum is removed with an intact mesorectal fascia, preserving tissue planes.

For low situated rectal tumours an abdominal perineal resection (APR) is preformed, where a complete TME of the rectal tumour is excised through access via the abdomen as well as the perineum. If the tumour is not situated to low, sphincter sparing surgery is possible with the APR, making an anastomosis possible. For high and midrectal tumours a low anterior resection is done where the most distal portion of rectum is spared and this makes intestinal continuity through an anastomosis possible. In older patients sometimes a Hartmann's procedure is preformed where a permanent stoma is created. It is common to establish a temporary colostomy or ileostomy to decompress a direct anastomosis, thereby decreasing the risk of anastomotic failure. For some patients, a direct anastomosis is not possible, which may result in a permanent stoma or these patients may later have intestinal continuity restored through surgery.

The importance of radiotherapy (RT) in rectal cancer is further discussed in Chapter 3.7.3.

3.6.2 Colon cancer

The surgical approach of a CRC in the colon depends on tumour localisation, but from the improved survival in rectal cancer that followed Heald's idea of

following the avascular planes and to excise the surgical specimen en-bloc has now changed the surgical technique also in colon cancer. The same ideas applied to colon cancer means that surgical dissection follows the mesocolic plane, with an intact mesocolon and with this an enveloping barrier of peritoneum and fascia of the surgical specimen with the major metastatic routes conserved within the tissue. The procedures usually preformed are right-side hemicolectomy, left-sided hemicolectomy or sigmoidectomy with for example a Hartmann's operation. Low sigmoidal tumours are a special group and can depending on their distal margin almost be viewed as rectal cancers.

Hoenberger et al presented improved five-year survival rates for colon cancer patients using the technique complete mesocolic excision (CME) with central vascular ligation (CVL) [29] based on Heald's principles of TME. The idea of CVL is to divide the supplying vessels at their origin. By using this technique more tissue and lymph nodes are retrieved [30] and this might correspond to the higher survival rates when compared to conventional colon surgery. However, further studies are needed in the area of colon cancer surgery, to clarify which method is the most optimal for the patient, both in regards to morbidity and mortality.

Furthermore, the acceptance for not resecting the primary tumour directly in patients with acute disease, such as ileus is now making grounds. The principle of treating the patient with a stoma in the acute phase if possible, to later enable good oncological surgery of the colon tumour by a colorectal surgeon is important for survival.

Other important considerations are whether the patient should have a stoma or if the resected parts of colon should be directly anastomosed.

3.7 Oncological treatment

The different oncological treatments are chemotherapy, radiotherapy, and biological agents. The regimens and the agents used are defined mainly by TNM stage, but also by the patient's general condition and planned surgical treatment.

3.7.1 Chemotherapy

Chemotherapy of CRC can be intended for *conversion therapy or down-sizing* where a non-resectable tumour is treated to shrink the tumour making surgical resection possible; *neoadjuvant therapy* where the tumour is resectable but the treatment is both administered prior resection and the rest is given after surgery to prevent disease recurrence; *adjuvant therapy* is given after resection to prevent recurrence of the disease and *palliative treatment* is used to slow the disease in patients not considered resectable or operable.

The most commonly used agents are 5-Fluorouracil (5-FU), a pyrimidine analogue that interferes with DNA replication in rapidly dividing cells. Fluorouracil is often combined with Leucovorine, a derivate of Folic acid that enhances the effect of 5-FU by the inhibition of thymidylate

synthase and this combination in known as FLV. Irinotecan is an agent that inhibits topoisomerase 1, which interferes with DNA replication and transcription. Irinotecan is usually combined with 5-FU and Leucovorine, a regime named FOLFIRI. Oxaliplatin is a platinum based agent, which forms inter- and intra- strand crosslinks of DNA, thereby leading to inhibition of DNA replication and transcription. Oxaliplatin is often combined with 5-FU and Leucovorine in the regime FOLFOX. In Sweden the modified regime FLOX is usually used instead, which allows the chemotherapy to be given in a peripheral vein in an shorter time than FOLFOX, which is administered into a central vein access for a longer time. Capcetabine is an orally administered pro-drug of 5-FU, which is activated to 5-FU via enzymatic steps, mainly in the cancer cells.

Patients with stage I disease have a 5-year survival of >95% and are therefore treated with surgery alone. Stage II patients are usually not treated with adjuvant chemotherapy, unless there are certain risk factors for recurrence. However, studies on this matter have been inconclusive [31,32]. There are findings that some patients in stage II benefit from adjuvant chemotherapy [33], and therefore much effort is put in trying to identify the patients that should be treated. Patients with stage III have a high risk for recurrence, a poorer survival and in general receive adjuvant chemotherapy. Palliative treatment of advanced disease in stage IV with chemotherapy, usually only results in a few months prolonged survival. But longer survival for up to 24 months can today be achieved with chemotherapy and modern agents. However, in stage IV disease there is a large group with limited metastatic disease such as patients with colorectal liver metastases (CLM). This group highly benefits from surgery of their metastases and will be further discussed in Chapter 4.

3.7.2 Biological treatment

During the last years, much effort has been made on developing biological agents for the treatment of cancer. The targets for these agents are identified key processes that can drive tumorigenesis.

Cetuximab is a chimeric mouse/human antibody directed against the epidermal growth factor receptor (EGFR), thus silencing the signal mainly in cancer cells with a mutated EGFR receptor, thereby turning the signals off that make these cells divide abnormally. In certain CRC however there are mutations of KRAS, a downstream protein in the EGFR signalling pathway. If KRAS is mutated, the patients do not respond to Cetuximab. Therefore, surgical specimens of CRC nowadays are screened for mutations of KRAS. Only patients with non-mutated or wild-type (wt KRAS) will respond to Cetuximab. Panitumumab is another fully human monoclonal antibody directed towards EGFR, thereby turning the EGFR signal off in wt KRAS cancer cells. Bevacizumab is a humanized monoclonal antibody that acts as an angiogenesis inhibitor by blocking Vascular Endothelial Growth Factor A (VEGF-A).

Though these new biological therapies provided much hope for the treatment of CRC as well as metastatic CRC, they have not yet revolutionised the survival of CRC patients.

3.7.3 Radiotherapy

Improved protocols for radiotherapy (RT) of rectal cancer in combination with refined standardized surgery, better staging and a multidisciplinary approach have greatly improved the survival of these patients. In general RT is used to decrease the risk of local recurrence after surgery by elimination of peripheral CRC cells in the peritumoural tissue. Patients who have received preoperative RT present with lower rates of recurrence, better overall and cancer-specific survival than patients that have not undergone preoperative RT [34]. In Europe RT is standard for T3-T4 tumours. RT can be given as short-term therapy to gain local control as mentioned above, long-term therapy to shrink a non-resectable tumour and also combined with chemotherapy (RTCT) for locally advance tumours to make surgical resection possible.

Blomqvist and Glimelius presented in 2008 a standardised view on how to treat rectal cancer [35]. The rectal cancer can be classified into three groups with different risks for recurrence and therefore different treatment strategies. The *good* rectal cancer with no negative prognostic features and low risk of local failure is treated with surgery alone and further with chemotherapy and/or RT if the findings in the surgical specimen indicate negative prognostic features. The *bad* cancer recti has increased risk of local failure (10-20%) with more advanced tumours and is treated with preoperative RT of 5x5 Gy followed by immediate surgery. The group of *ugly* rectal cancer with locally advanced tumours has the highest risk for local recurrence (20-100%) and is treated with either long-course low fraction RT of 40-50 Gy with combined chemotherapy (RTCT) or RT 5x5 Gy with delayed surgery in patients not fit for RTCT. The intention is to induce downstaging and downsizing of the tumour, and make successful surgery possible in an initially non-resectable tumour. The addition of neoadjuvant chemotherapy in advanced rectal cancer has been shown to induce downstaging and decrease local recurrence and also improve survival [36,37].

Chapter 4: Colorectal liver metastases

The liver is a common site of metastasis of many tumours, in particular tumours of gastrointestinal origin due to the portal drainage. Patients with isolated CLM can however undergo surgical resection and achieve long-term survival, compared to liver metastases (LM) of other origin. This is mainly due to the fact that other tumours more often present with a disseminated and extrahepatic disease in addition to the LM. However, there are selected cases of LM from cancers of other origin that might benefit from liver resection due to metastasis restricted to liver only. Metastatic liver surgery has increased during the last decades due to the improved surgical techniques and the possibility to perform curative intended surgery on patients with more advanced hepatic tumour burden. Today, patients with resectable CLM, and even isolated lung metastases are subjects to successful surgical intervention, making long-term survival in these patients possible.

4.1 Incidence and survival

In Sweden almost 6000 patients are diagnosed with CRC yearly. About half of these patients will present with or develop colorectal liver metastases (CLM). Of these approximately 15-20% are eligible for curatively intended surgical resection of their CLM. Untreated, the survival is only 6-9 months [38], but chemotherapy can prolong survival.

4.2 Pathogenesis

The metastatic spread of CRC cells is mainly considered to be haematogenous through the drainage of the gastrointestinal tract through *v. porta*, where blood from a tumour is drained directly into the liver sinusoids. Thereby circulating CRC cells enter the liver via the portal system. The metastatic process is however a complex multi-step sequence and is discussed more extensive in chapter 6.

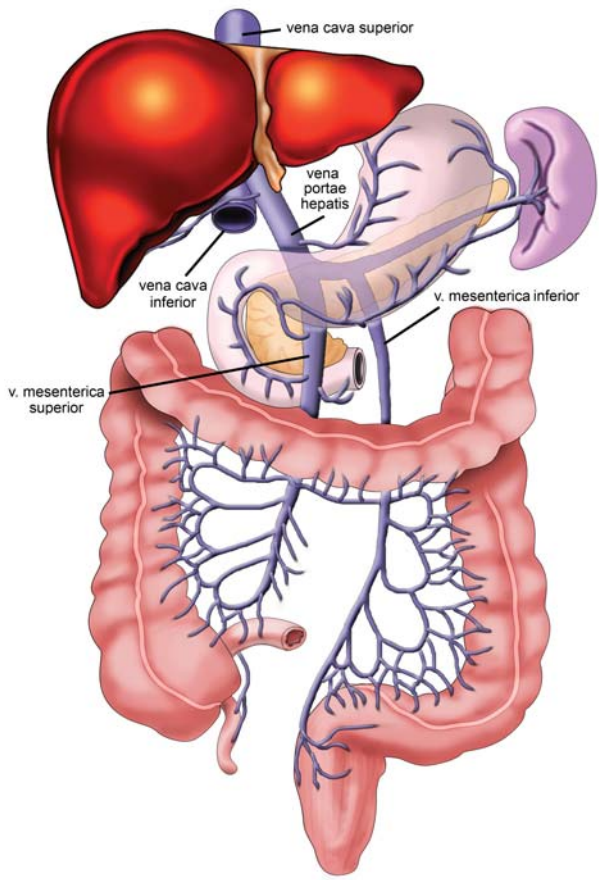

Figure 7
The vena porta drains the gastrointestinal tract. The liver sinusoids are directly exposed to the venous blood from the colorectum, where CRC cells can spread via the hematogenous route. © G. Andersson 2013

4.3 Diagnosis and Staging

About 1/3 of patients with CRC present with synchronous LM, thus the metastasis are discovered at the time of diagnosis of the primary tumour. The remaining 2/3 presents with metachronous LM, which are often discovered by routine follow-up with CT scans after surgery of the primary tumour. The standardized screening for metachronous CLM of CRC patients after the surgery of their primary tumour enables for early detection of CLM, thereby making surgical resection of CLM possible.

4.4 Surgery and oncological treatment

The first liver-resection is thought to have been preformed during the 1800-century. In the beginning of the 1900-century small advances were made in the area, especially by J. Hogarth Pringle who in 1908 described digital compression of the hilar vessels to control traumatic bleeding from the liver. As previously mentioned the understanding of the functional anatomy of the liver provided knowledge that enabled the first anatomic right hepatectomy by Lortat-Jacob in 1952. Initially liver surgery was associated with high mortality, but tremendous advances have been made since. Today, liver surgery has gone from larger surgical resections (lobectomy) to parenchyma-sparing surgery (segmentectomy and metastasectomy). About 70% of the liver parenchyma can be resected, leaving at least 30% for sufficient liver function. Studies of CLM have shown that a resection margin of 4 mm is sufficient, and that there are no tumour cells beyond this border, which explains why such good results can be achieved with parenchyma-sparing surgery [39]. Factors that affect the resectability are; size and number of CLM, localisation of CLM, volume and function the of residual liver, the patient's condition and findings of extrahepatic disease. Isolated lung metastases are not a contradiction to liver surgery, since these can be resected successfully as well.

Radiofrequency ablation (RFA) is a complement to conventional surgery, where a probe is inserted in the tumour and heat is generated to destroy the cancer cells. The efficacy of this treatment is however not optimal and many patients develop local recurrence at the border of where the RFA was performed. There are no randomised, controlled trials comparing RFA and surgery, thereby making comparison impossible [40]. Microwave ablation is a similar technique where microwaves are used to generate a heat-mediated cell-destruction. Ablation is today mainly a complement to conventional surgery or an alternative in elderly patients not fit for major surgery. Many techniques for the treatment of CLM have been studied, but still conventional surgery is the main treatment and provides the best results. The 5-year survival of patients resected for CLM varies between 30-50%, and is highly dependent on patient selection [41,42]. Many prognostic factors have been identified with the following factors associated with a poor outcome: male gender, elevated CEA, multiple CLM, large CLM, bilobar involvement, stage of primary disease, regional nodal metastases, synchronous CLM and a short disease-free interval (time from CRC to CLM)

[43,44].

Many patients develop postoperative recurrence after surgery of CLM. Adjuvant treatment with chemotherapy (FOLFOX) is therefore often used. The rationale for this was to extrapolate the well-documented benefits from adjuvant treatment for primary CRC to CLM. Mitry et al. reported that patients treated with adjuvant chemotherapy displayed a longer progression-free survival (PFS) than patients treated with surgery alone [45]. However both randomized controlled trials on which Mitry´s report are based on, had to be closed in advance due to slow recruitment of patients, thereby making statistical power of these studies questionable.

In recent years there has been a growing trend in administering preoperative as well as postoperative chemotherapy for these patients (referred to as *perioperative* or *neoadjuvant* treatment). However, the clinical benefit of this is unclear. One randomised study, the EORTC showed that CLM patients with 1-4 metastases when given a perioperative regimen with FOLFOX had a significant longer 3-year progression-free survival (PFS), increasing from 28.1% to 36.2%, when compared to surgery alone [46]. However, during the follow-up of this study, no difference in overall survival was seen, thereby making the clinical gain of the perioperative treatment questionable. Furthermore, an increased frequency of postoperative complications was reported. There are also more factors to consider when it comes to perioperative/neoadjuvant chemotherapy. One has to consider missing the window of resection due to tumour progression or a complete tumour response, making affected areas difficult to visualize during surgery [47]. The European colorectal metastases expert group recommends perioperative treatment if there is a high risk of recurrence, with multiple CLM, CLM larger than 5 cm, synchronous CLM, lymph nodal involvement or high CEA [48]. There is no evidence that patients with a single lesion benefit from perioperative chemotherapy [49]. Thus, there is a great need for a prospective, randomized phase III trial comparing adjuvant vs. perioperative/ neoadjuvant therapy in patients undergoing liver resection for CLM.

Patients with non-resectable advanced CLM can be treated with conversion therapy, which might make liver resection possible, if the metastases shrink in response to given therapy. Patients who respond with substantial tumour regression can then become resectable. A study in 2004 showed that patients with initially unresectable CLM could undergo surgical resection with a reported 5-year survival of 33 % [50]. However, only 12.5 % of patients that were given a conversion regimen responded, allowing for subsequent liver resection, and many of the patients underwent repeated surgeries (both hepatic and extrahepatic).

Additionally, more advanced techniques are available for patients with advanced hepatic disease such as portal embolization, surgery in combination with ablative treatment and two-stage hepatectomy. Also in patients with rectal cancer, the approach ”liver first” is now accepted, when the CLM are first resected and then later the locally advanced primary tumour. Liver-metastatic surgery is a rapidly expanding field and more and more patients are considered for resection due to better treatment options.

Chapter 5: The stroma and the basement membrane

The stroma is defined as the connective and functionally supportive framework of an organ. It consists of inflammatory cells, vascular cells and fibroblasts, but also extracellular matrix (ECM) proteins such as collagens, fibronectin, glycosaminoglycan's and proteoglycans. The basement membrane (BM) is a specialised form of ECM, which underlies most monolayered epithelial and endothelial cells in the human body [51]. The BM separates the epithelial cells from the tissue underneath and is important for maintaining tissue integrity and polarisation of epithelial cells. The BM provides binding sites for cell adhesion receptors, which are important for cellular processes such as growth, survival, migration and apoptosis.

Approximately 50 different proteins compose the BM [52]. The most predominantly found proteins are type IV collagen, laminin, nidogen and perlecan. Type IV collagen and laminin self-assemble into sheet-like structures and provide the structural scaffold of the BM. Nidogen and perlecan can cross-link the type IV collagen and laminin sheets, and thereby strengthen the structure of the BM. The composition of BM differs between the tissues in the body and is related to function. An example of this is the specialized BM found in the kidney glomeruli, which allows for filtering of the blood. Cells adhere to the BM mainly by integrin's, a family of cell surface receptors.

5.1 Collagens

Collagens are a class of proteins that are the main component of connective tissue and the most abundant protein in mammals, accounting for 25-35% of the total protein content in a body. There are over 30 known collagen types. Collagens are characterized by repeats of a unique amino acid (aa) sequence where every third aa is a glycine (the Gly-X-Y motif). This allows the protein to trimerize into triple helical structures. Collagens are divided into two groups depending on their structural assembly, namely fibrillar collagens (such as type I and III collagen) and non-fibrillar collagens (such as type IV collagen). Fibroblasts are the main producers of collagen.

5.2 Type I collagen

Type I collagen is the most abundant ECM protein in the body and is composed by a trimerization of two α1- and one α2-chain. Bone, tendons and ligaments are all tissues rich in type I collagen, and this ubiquitous protein provides binding sites for numerous molecules [53]. It is a fibrillar collagen and over 300 variants of type I collagen mutations related to connective tissue diseases have been described [53]. One example is a special form of Ehler-Danlos syndrome (the artrochalasi type – type VII A and B), which is caused by mutations in the gene coding for the α1 chain (COL1A1).

Type I collagen is synthesized as a soluble pro-collagen form containing globular C- and N-propeptides, joined to the respective ends at the triple helix. When the pro-collagens are secreted from the cell, the propeptides are cleaved by proteinases, and the collagen monomers then self assemble into a fibril. A fibril is composed of microfibrils (5-mer bundles of monomers) overlapping each other.

5.3 Type III collagen

Type III collagen is the second most abundant collagen in human tissues, and is found mainly in tissues with elastic properties, such as skin, blood vessels and various internal organs. Type III collagen is a homotrimer composed of three α1-chains, and resembles other fibrillar collagens in its structure and function. Its elastic properties may be due to disulphide bonds, and the fact that there is no lysyl oxidase-dependent cross-linking in the C-terminal end [54]. This protein is synthesized as a procollagen similarly to type I collagen.

Mutations of type III collagen (COL3A1) makes tissues and especially arteries frail and causes the most severe form of Ehlers-Danlos syndrome (vascular type- type IV), which affect the internal organs, joints, skin and arteries with a subsequent high risk of aortal rupture and death.

5.4 Type IV collagen

Type IV collagen is a non-fibrillar collagen, and self-assembles into suprastructures of sheet-like networks. This is the most abundant BM protein in the human body. The protein rises from combination of six different α-chains (α1-α6), which trimerize (**Figure 8**). Each chain is the product of a gene (COL4A1-COL4A6). The α-chain consists of a non-collagenous domain (NC1-domain) at the C-terminal, an intermediate collagenous domain (containing the Gly-X-Y motifs) and at the N- terminal a 7S domain.

In mammals, the genes of type IV collagen are arranged in three pairs on different chromosomes, displaying a head-to-head organization. COL4A1 and COL4A2 are located on chromosome 13, COL4A2-COL4A3 on chromosome 2 and COL4A5-COL4A6 on chromosome X [55]. Each gene pair shares a bidirectional promoter, however the regulation of the gene expression is not clear. Normally, the mRNA levels of type IV collagen vary, but the ratio of translated α-chains remains the same. The explanation for this is thought to be due to a regulation on both a translational and posttranslational level [55]. Protomers begin to form in the Golgi apparatus, where the NC1-terminal domain initiates the triple helical structure in a zipper-like manner; Protomers are secreted and then self-assemble into the type IV collagen suprastructure. Here four 7S-domains join each other and stabilize covalently and two NC1 domains form head-to-head interactions [51].

Type IV collagen seems to be an essential protein as most mutations are thought to produce lethal phenotypes [56]. The combination of different α-chains give rise to subtypes of type IV collagen with different features.

Protomers of α1α1α2 are found in all BM, while α3α4α5 are found in the BM of the kidney, lung, testis and eye, and the α5α5α6 protomer is mainly present in skin, smooth muscle and the kidney.

Alport´s syndrome is a disease caused by mutations in type IV collagen α5-chain gene. The α5-chain is needed in the specialized BM of the kidney and the ear, thus the disease is characterized by BM malfunction giving rise to glomerular nephritis and hearing loss [57].

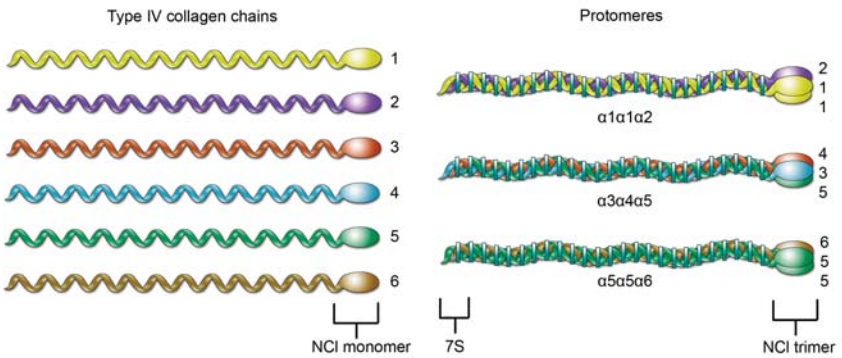

***Figure 8**. The 6 α-chains of type IV collagen and its assembly into protomers. The protomers self-assemble into sheet-like structures extracellularly.*

5.5 Stroma in cancer

For many years the tumour cell has been the predominant area of the focus in the scientific community. During the last two decades however focus has shifted towards the microenvironment of the tumour, with emerging research areas such as angiogenesis, inflammation and stroma. The stroma in a tumour consists of the ECM proteins and the non-malignant cells of the tumour (fibroblasts, inflammatory cells and the vascular cells). The stroma often substantiates a major part of the total volume of a solid tumour [58]. Tumour stroma is also referred to as the reactive or desmoplastic stroma, and displays increased numbers of fibroblasts and capillaries, as well as an abundant deposition of type I collagen and fibronectin [58]. The composition of the tumour stroma resembles granulation tissue, whereby Dvorak formulated the seminal description of tumours as wounds that do not heal [59].

The process of establishing a tumour stroma is initiated by the cancer cells by the release of stromal growth factors (bEGF, VEGF, PDGF, EGF, interleukins, TGF-β and many more). This subsequently leads to the activation of stroma producing fibroblasts [60], which disrupts and alters normal tissue homeostasis [61]. In addition, the processes of angiogenesis [62] and inflammation [63] are initiated. The process is thereafter reinforced by the activation of even more stromal cells. Components of the stroma such as cancer associated fibroblasts (CAFs), tumour-associated macrophages (TAMs) and endothelial cells play many important roles in the progression, growth and metastasis of tumours [64-66] [67].

The tumour stroma is constantly remodelled by the action of different proteases [68] in the tumour microenvironment. This facilitates for invasion and migration of cancer cells. The exposure of ECM fragments due to breakdown of the BM also provides with signals that can act in an angiogenic or anti-angiogenic manner [52]. There is evidence that an abnormal stromal compartment can act in a mutagenic way, thus malignifying normal cells [69,70] and that a normalized stroma can reverse malignant cells [71] [72]. Thus, the stroma is undoubtly an important factor in tumour progression. The stromal remodelling, in the process of cancer is illustrated in **Figure 9**.

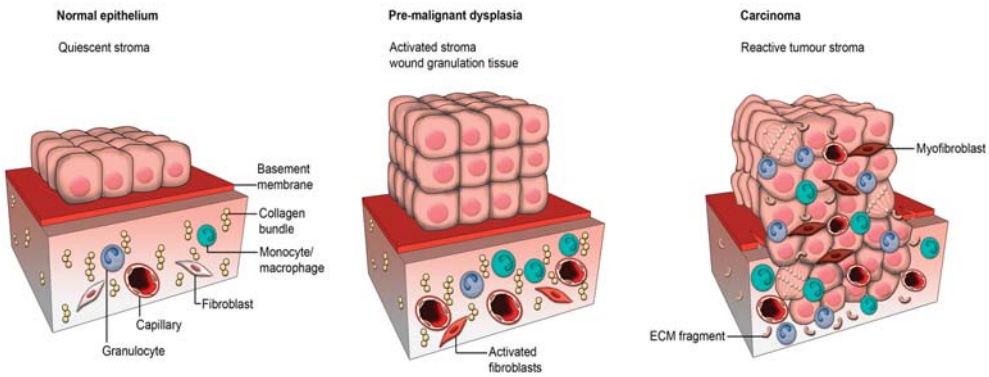

Figure 9. *The normal stroma with an intact BM, the stroma activated in premalignant dysplasia and the loss of BM integrity and the stromal remodelling in an invasive cancer.* © *G. Andersson 2013*

Chapter 6: The stroma in CRC and in CLM

This chapter will focus on the stroma in CRC and CLM with a special focus upon the ECM components. First, the normal stroma and ECM composition is discussed for each organ, and is then followed by the stroma of CRC and CLM. The term desmoplastic reaction (DR) is referring to the reactive stroma of a tumour and is characterized by a degradation of the type IV collagen rich BM, and a deposition of fibrillar collagens [73,74].

6.1 Stroma in normal colorectum and in CRC

In the normal mucosa of colon and rectum, type I and III collagens are found predominantly located in the interstitial stroma, and type IV collagen in the BM of the mucosa and blood vessels [75].

Type IV collagen is the major component of the BM and its constitution in the normal colonic mucosa has been investigated. The α1-, α2-, α5-, and α6-chains are found in the BM delineating mucosal epithelium and the gland crypts, and the α3- and α4 – chains are found in BMs of the luminal surface epithelium, but the BM integrity is lost in CRC [76].

Additionally, in CRC the loss of type IV collagen staining (α1 and α2-chain) is related to poorer differentiation of the tumour. The cause of type IV collagen degradation is related to the activity of MMPs in the tumour microenvironment (MMP-2 and MMP-9)[77]. During the process of CRC development the normal BM is degraded, and this defines when a non-invasive carcinoma in situ (CIS) becomes an invasive cancer [78-80].

In CRC a pronounced DR has been related to poor prognosis [81], but these results are inconclusive and have not been reproduced in *in vitro* studies. In a recent gene expression study, CRC was classified into different subtypes, namely a mucinous subtype, a high-stroma subtype and also a low-stroma subtype. Interestingly, patients with low-stroma subtype tumours had significantly better survival compared to patients with the other subtypes [82]. Additionally, it has been shown that CRC with a high proportion of stromal myofibroblasts have a significantly worse disease-free survival, when compared to CRC with less myofibroblasts [83].

6.2 Stroma in normal liver and in CLM

The ECM in the liver provides structural bond between cells, induces and maintains cell polarization, allows intercellular communication and also affects gene expression and cellular differentiation. Collagens constitute about 5-10% of the normal liver stroma, but considering its size this only makes up a small amount compared to other tissues. Many different collagens are found in the liver (such as type I, III, IV, V, VI and XVIII collagen), but the major types being type I and III collagen, and these two proteins make up 95% of all collagen found in the liver. Type I collagen is predominantly located in portal tract matrix, hepatic veins and at the point of inflection in the hepatic cords. Type III and IV collagen are also found in the portal tracts and in the space of Disse. Other major proteins are glycoproteins and proteoglycans. Laminin is the major glycoprotein in normal basement membranes and interacts with type IV collagen.

The process of metastasis is a multi-step process characterized by cell detachment of the primary tumour, cell migration, invasion of host tissues, extravasation and finally tumour cell adaptation. The British surgeon Stephen Paget already in 1889 presented the theory of seed and soil where he compared plant seeds with metastatic tumour cells that need a permissive soil to grow and thereby establish metastases. The process of liver metastases from the gastrointestinal channel is anatomically explained by the drainage of v. porta. The liver is however also a major metastatic site for many other tumours and it is a well-established fact that the “soil”, and not only the vascular anatomy play an important part in the metastatic process.

The stroma in primary CRC has been far more investigated than the stroma in CLM. Gulubova et al. reported findings of increased tenascin expression in both CRC and subsequent CLM [84]. Type I, III and IV collagen has been reported as the major collagen components of the DR seen in CLM, where also a rich infiltrate of α-SMA positive myofibroblasts was present [85]. Metastatic growth patterns of CLM with a special emphasis on stroma have also been examined and are discussed separately in the following chapter.

The source of ECM deposited in CLM is not established. Hepatic Stellate Cells (HSC), hepatocytes and sinusoidal endothelial cells all possess the ability to produce ECM in the liver [86-88]. These functions have been extensively studied in the field of fibrotic liver disease. Mainly HSCs are thought to rediffrentiate into α-SMA positive myofibroblasts that are responsible for the ECM deposition seen in fibrotic liver disease but may also produce the DR in seen in CLM. In 2007 Mueller et al. described histological findings supporting that the α-SMA positive myofibroblasts seen in CLM could originate from liver fibroblasts [89].

Also, it has been shown that certain cancer cells have the ability to produce their own BM proteins such as type IV collagen [90] and that these cancer cells have the potential to bind to type IV collagen [55]. In a liver metastatic cell-line of murine lung carcinoma in which the self-production of type IV collagen was blocked, lost its liver-metastatic potential [91], implicating the possible need of type IV collagen to establish liver metastases. Type I collagen on the other hand, has been has been shown to induce chemoresistance, proliferation and growth of CRC cells [85].

6.2.1 Metastatic growth patterns in CLM

In 2001 Vermeulen et al. presented three different growth patterns of CLM based on histology [92]. These were referred to as the desmoplastic, pushing and replacement growth pattern. The desmoplastic growth pattern, seen in 42% of the cases, was characterized by the presence of a thick desmoplastic reaction (DR) separating the tumour cells from normal liver parenchyma, with a rich inflammatory infiltrate present in this area. In the pushing growth pattern (observed in 46% of the cases) the tumour cells pushed the normal parenchyma away, with only a minimal DR and little inflammation present. The replacement growth pattern was more unusual (in 12% of cases) and was characterized by tumour cells directly growing within the normal reticular architecture of the liver with no DR and little inflammation present.

There are studies indicating that patients that have a CLM with a fibrous capsule have a better prognosis than patients without this feature. In 2001 Lunevicius et al. classified CLM histologically into “capsulated” or “non-capsulated”, with the finding that capsulated CLM have a better prognosis with fewer hepatic recurrences compared to the non-capsulated CLM [93]. Similar findings have been described in hepatocellular cancer (HCC). The findings in HCC was also the base for a study in 2000 where Okano et al. presented a significantly better 5-year survival in resected CLM patients with a histologically verified capsulated metastasis [94]. Rajaganeshan et al. published a work where the morphological pattern in both primary CRC and the subsequent CLM was assessed in relation to angiogenesis [95]. They found that a CRC with an infiltrative primary CRC tended to form infiltrative (or non-capsulated) CLM, and that primary CRC with a non-infiltrative margin tended to form encapsulated CLM.

The focus of this thesis has been the stroma and its constitution of collagens in CRC and CLM; different growth patterns of CLM, clinical relevance of the ECM composition as well as the stroma as a source of

potential tumour markers for CLM. The aspects of tumour markers are discussed in the following chapter.

Chapter 7: Circulating tumour markers in CRC and CLM

The optimal tumour marker should possess as many as possible of the following criteria:

- Easily accessible in body fluids or tissue
- Show optimal sensitivity and specificity
- Reflect the tumour burden
- Be analysed with stable and easily reproducible methods

Depending on the clinical situation the marker can work as a screening marker, a diagnostic marker, a prognostic marker or a surveillance marker. Currently, there is no tumour marker that fulfils all the above criteria, but there are many useful ones. Many tumour markers have been characterized, but few have reached clinical practice, since they do not meet the criteria required. Special consideration should be given to whether the marker will be used for screening in a general, unselected population, or if it will be used in well-defined high-risk population. There are no optimal tumour markers for CRC or CLM today. Additionally, very few studies have evaluated tumour markers in larger well-defined CLM materials only.

7.1 CEA

Carcinoembryonic Antigen (CEA) is a surface anchored glycoprotein of 180-200 kDa and is normally expressed in the fetus and the production stops before birth. CEA is related to the family of adhesion molecules such as intercellular adhesive molecule 1 (ICAM-1) and major histocompatibility antigens (MHCs) [96,97].

The circulating and the expressed tissue levels of CEA in CRC seem to correlate with the differentiation of the CRC tumour, with well differentiated tumours producing elevated levels of CEA and poorly differentiated cancers presenting with low levels [98,99]. The circulating levels also correlate to a degree to increasing tumour stage, with the highest levels found in stage IV disease (65%)[100]. CEA is however not specific for CRC and is found elevated in many other conditions such as benign liver disease, pancreatitis, other tumour forms and in smokers [101-103]. Additionally, not all CRC or CLM produce CEA.

In stage I and II CRC, CEA has a sensitivity of 36% and specificity of 87%, when using a cut-off level of 2.5 μg/l [104]. The clinical cut-off level for CEA in Sweden is set at 5 μg/l. Due to its suboptimal sensitivity and specificity CEA is not used as a screening marker. It can be of help as a diagnostic marker if found elevated (with a cut-off of 2.5 μg/l). The percentage of patients with increased levels ranging from 28% in stage I,

45% in stage II, 75% in stage III and 84% in stage IV disease. If a cut-off level of 5 μg/l is used, the corresponding numbers are as follows: stage I 3%, stage II 25%, stage III 45% and stage IV 65% [100]. Therefore, CEA is not optimal as a screening or diagnostic marker for CRC, but high levels are related to a poor survival [38,105], making its prognostic function more valuable.

CEA is found elevated in about 50-60% of patients with CLM, and thus has its main role in surveillance after surgery of primary CRC [106] [107]. CEA today is a standard test in patients with CRC, but is most beneficial for surveillance if found elevated at the time of diagnosis of the CRC [108]. However, measuring CEA has not replaced any routine follow-up examinations of CRC patients, such as CT-scans, since the majority of patients with recurrent local CRC and at least 40% of the patients with CLM will not have elevated levels. Additionally, the CEA monitoring seems to have only a modest effect on over-all patient outcome [109].

7.2 Stroma-derived tumour markers

This section will focus on circulating tumour markers derived from the stromal compartment and mainly from the ECM.

7.2.1 Type IV collagen

Circulating levels of type IV collagen are used for the monitoring of chronic liver diseases, since the levels seem to correlate with the hepatic expression of this protein and grade of the disease, and decrease in response to therapy [110]. Type IV collagen has previously been investigated as a tumour marker for CLM [111], as well as in primary and metastatic hepatocellular carcinoma [112] with promising results. Levels of type IV collagen have also been analysed in peritoneal fluid from patients with adenocarcinoma of the gastrointestinal tract, and high levels correlated to peritoneal dissemination and a poor survival [113]. Öhlund et al. have found that circulating type IV collagen is elevated in patients with pancreatic cancer [90]. Normal levels for healthy controls have been established, with no difference in concentration for sex or age between 20-60 years [114]. For ages under 20 and over 60 years slightly higher levels were seen.

7.2.2 Other BM related protein fragments

Endostatin is a fragment derived from cleavage of type XVIII collagen and is also found in the BM. It has anti-angiogenic properties and is an endogenous angiogenesis inhibitor [52,115]. Patients with CLM display higher circulating levels of endostatin than healthy controls, and patients with hepatic recurrence after liver surgery have increased levels, compared to patients with no recurrence [116]. Circulating endostatin levels in patients with CLM, HCC and gastric carcinoma was found elevated when compared to controls [117], implicating the potential of this ECM protein fragment as a tumour marker. Endostatin has also been found elevated in patients with pancreatic

cancer [118].

Laminin is also a BM protein. Circulating levels of laminin was found to be higher in patients with CRC and even higher in patients with CLM and high levels correlated to a poor survival [119].

7.2.3 VEGF

Vascular endothelial growth factor is a family of proteins that stimulates vasculogenesis and angiogenesis. It is produced by many cell types, but in cancer stromal cells are the likely main source of VEGF [120].

Circulating VEGF-C was found to correlate to lymph node metastasis in CRC and a poor survival [121]. In a study analysing several potential circulating tumour markers in patients with CRC, VEGF in combination with other markers (CA19-9, CEA) showed promising results, but not by itself [122]. A study in 2010 found that circulating VEGF was significantly higher in CRC patients compared to healthy controls, and that this correlated to tumour size and CEA levels, and was a factor for poor survival [123]. There have been numerous studies on tissue expression of VEGF. In CRC tissue expression of VEGF correlates better with disease-free survival than conventional clinicopathological factors [124].

7.3 Other circulating tumour markers

Many different circulating tumour markers have been evaluated in CRC and CLM, but none have yet met the criteria of sensitivity and specificity described above, and none has proven to be better than CEA. Arginase, the last enzyme in the urea cycle, was found to be a potential tumour marker for CLM [125]. Carbohydrate antigen or Cancer antigen (CA 19-9) has been found elevated in many gastrointestinal cancers including CRC [126,127]. CA242 and CA 72-4 are two other markers resembling CA 19-9 that have been investigated as tumour markers in CRC [128] [129]. Tissue polypeptide antigen (TPS) is a soluble epitope from cytokeratin 18, and has also been evaluated in CRC [127].

II. Aims

General aims

The aim of this thesis is to study the stroma and especially the extracellular matrix (ECM) compartment in colorectal cancer (CRC) and in colorectal liver metastases (CLM). The thesis focus on the tumour biological implications of the tumour stroma and also on the characterization of new tumour biomarkers for CLM, derived from the stromal compartment.

Specific aims

- To investigate the expression pattern of the α1(IV)-α6(IV) chain of type IV collagen in normal liver and in CLM.
- To measure circulating levels of type IV collagen in patients with primary CRC in stages TNM stage I-III (Duke´s A-C).
- To measure circulating levels of type IV collagen in patients with CLM at the time of diagnosis, during treatment with palliative chemotherapy and at the time of disease progression and to compare this with circulating levels in healthy controls.
- To grade the hepatic tumour burden of CLM in each patient and correlate this with the circulating levels of type IV collagen.

(Paper I)

- To compare whether the stromal composition in primary CRC that never metastasised differs from the stroma in primary CRC that metastasised to the liver.
- To study the expression of type I, III and IV collagen in non-metastatic CRC, liver-metastatic CRC and its subsequent CLM.
- To investigate earlier described growth patterns of CLM and to characterize the collagenous components of these growth patterns.
- To evaluate if the growth pattern of CLM can be predicted from a stromal analysis of the primary tumour.
- To relate the stromal composition of non-metastatic CRC, liver-metastatic CRC and its subsequent CLM to clinical parameters and overall survival.

(Paper II)

- To measure preoperative circulating type IV collagen levels in patients with resectable CLM.
- To measure postoperative circulating type IV collagen levels in the patients that underwent surgical resection, and relate this to recurrent disease or not.

- To compare circulating type IV collagen to the conventional tumour marker CEA.
- To classify the metastatic growth patterns in the same patients and relate this to the levels of circulating type IV collagen.

(Paper III)

- To investigate if CRC cell lines can produce type IV collagen endogenously and in a liver stromal context.
- To develop a novel organotypic liver metastatic model for the three dimensional study of liver metastases with a special emphasis on the stromal context
- To analyse circulating type IV collagen levels in liver metastases of different embryological origin and to compare this with levels in benign liver lesions.

(Paper IV)

III. Materials and methods

Chapter 8: Patients and clinical data

Patients with CRC, CLM and liver metastases (LM) of other origin than CRC were admitted to the Department of Surgery at Umeå University Hospital for surgical treatment (paper I-IV) or palliative chemotherapy (paper I).
Patients with LM considered resectable and with no signs of spread disease (other than resectable lung metastases or primary CRC planned for surgery after liver resection) underwent surgical liver resection and/or radiofrequency ablation (RFA) (paper I-IV).

Patients with non-resectable CLM (n=15) (Paper I) were treated with hepatic artery occlusion for 16 hrs followed by intra-portal administration of 5-FU for five days every sixth week (n=7) or intra-arterial infusion of 5-FU+ Leucovorine for two days every second week (n=8) according to protocols earlier described [130]. Clinical data was collected from patient charts. CEA levels were retrieved from patient charts for the corresponding time of the plasma sample used to analyse stromal markers. The CEA samples were analysed in an accredited laboratory. During 1998-2002 the analysis was undertaken with the AxSYM CEA method (Abbot) and from 2002-forward by the Immulite 2000 Method (Siemens). The methods agreed well at transition from AxSYM to Immulite 2000. Survival data was obtained from clinical records. The hepatic tumour volume of CLM was estimated from preoperative CT-scans and MRI (Paper I). 27 patients in paper II were also included in paper III, where all histological grading was performed again with blinding of previous results.

Chapter 9: Patient samples

9.1 Plasma samples of patients with CRC, CLM, other LM and benign liver lesions

Plasma samples from patients with non-resectable CLM treated with palliative chemotherapy (intra-arterial or intra-portal regime) were collected at the time of diagnosis, during treatment and until disease progressed (Paper I). Criteria for progression were radiological finding of tumour progression, findings of extrahepatic spread and clinical findings supporting tumour progression. The time of progression was set by two independent clinicians.

Plasma samples from CLM patients undergoing surgical resection and/or RF ablation with curative intent were collected before surgery (2 weeks-1 day prior liver surgery) (paper III and IV) and postoperatively (6-42 months after surgery) (paper III). Plasma samples from patients with CRC (n=32) (TNM-stage I-III) were collected prior to surgical and oncological treatment. All samples were stored in -80° C until analysis.

9.2 Tissue samples of patients with CRC, CLM, other LM and benign liver lesions

Fresh-frozen tissue from CLM was cut out in a standardized way representing both tumour and the tumorous border as well as normal liver parenchyma by the responsible surgeon (paper I and IV). The tissue samples were snap frozen in liquid nitrogen directly in the operating theatre and stored in -80°C until analysis.

Paraffinated tissue sections from patients that underwent surgical resection for CRC (n=79), CLM (n=104), other LM (n=31) and benign liver lesions (n=5) were retrieved from the biobank at the Department of Pathology at Umeå University Hospital (paper II-IV).

9.3 Plasma samples from controls

Blood samples from patients undergoing surgery or endoscopic treatment for benign disease (cholecystectomy n=4, ERCP n=3 and colonoscopy n=1) were used as controls in paper I. In paper III, samples from healthy individuals were used (n=118). These 118 individuals donated blood samples during population based voluntary health controls, and the samples were stored in a prospective biobank of Västerbotten County Council and Umea University. All samples were stored in -80°C until analysis.

9.4 Tissue samples from controls

Tissue of normal liver parenchyma was collected from patients undergoing surgery for suspected malign liver lesions where macroscopically normal areas of tissue were cut out by the surgeon. The tissue was then snap frozen in liquid nitrogen and stored in -80° until analysis or experiment. In paper I and IV tissue samples of normal liver was used from 6-10 patients.

Paraffinated sections of normal colorectum (n=2) and normal liver (n=2) were retrieved from the biobank at the Department of Pathology at Umeå University Hospital.

9.5 Ethical approval

The Regional Ethical Review Board in Umeå approved the studies (project number 05-028M and 09-175/2009-1378-31). Informed written consent was obtained from all patients.

Chapter 10: Protein detection

10.1 ELISA and Luminex assays

In paper I, III and IV an enzyme-linked immunosorbent assay (ELISA) was used to measure the circulating levels of type IV collagen. A commercially

available ELISA-kit (Serum Collagen IV EIA, Argutus Medical), where two monoclonal antibodies (clones 4H12 and 1D3) directed against the 7S and collagenous domain of type IV collagen was used [131]. For measuring circulating CEA levels in controls (paper III) we used a commercially available Luminex based assay (Milliplex MAP Human Circulating Cancer Biomarker Magnetic Bead Panel, Millipore, Billerica, MA, USA). Control samples tested were within the normal range defined by the manufacturer.

10.2 Tissue and cell staining

The different techniques of tissue (paper I-IV) and cell staining (paper IV) are here briefly reported. The primary and secondary antibodies used in this thesis are presented in **Table 2**. For each antibody a positive control was preformed on tissue known to express the antigen investigated. Negative controls for each antibody were done with application of only the secondary antibody on the tissue slides. Primary and secondary antibodies used are presented in **Table 2**.

10.2.1 Immunohistochemistry and chemical staining

Immunohistochemistry and chemical staining was preformed on five μm sections of paraffinated tissue. For general histology examination a chemical routine stain with haematoxylin and eosin (H&E) was used (Paper I-IV). A reticular chemical stain was performed to visualise the reticular fibers (paper II-III). For the staining of type I and III collagen the chemical Picro-Sirius Red stain was used (Poly-Sciences, Inc.). This stain differentiates between the two different collagens using polarized light (Paper II).

Immunohistochemical staining for type I and IV collagen (Paper II and IV) was preformed using the Ventana Benchmark automated immunostainer (Ventana Medical Systems). The primary and secondary antibodies used are illustrated in **Table 2**. The secondary antibody was linked to peroxidase followed by diaminobenzidine tetra hydrochloride (DAB) as a chromogen.

10.2.2 Immunofluorescence

Immunofluorescence (IF) staining (Paper I and IV) was performed on five μm frozen tissue sections and on cells cultured on Falcon tissue slides (BD Biosciences) according to protocols earlier described [132]. Sections and cells were fixed in cold acetone (-20°C) or paraformaldehyde (PFA) for 10 minutes followed by air-drying. The slides were rinsed in phosphate buffered saline (PBS) and then blocked in a blocking solution of PBS with 3% Bovine Serum Albumin (BSA) for one hour. After this the slides were incubated with the primary antibody of choice with incubation for one hour. Then the slides once again were washed three times with PBS followed by the addition of a fluorophore-conjugated antibody and incubated under light-free conditions for one hour. The slides were then washed with PBS and the Vectashield anti-fade mounting medium containing DAPI (Vector Laboratories, App Imaging) was applied followed by cover slippage. All incubations were performed at room temperature.

Antigen	Manufacturer	Code	Type	Dilution	Fixation	Application	Paper
Primary antibodies							
α1(IV)NC1'	Wieslab	MAB1	Monoclonal mouse IgG	1:75	aceton or PFA	IF	I
α2(IV)7S	Chemicon	MAB1910	Monoclonal mouse IgG	1:400	aceton or PFA	IF	I
α3(IV)NC1'	Wieslab	MAB3	Monoclonal mouse IgG	1:75	aceton or PFA	IF	I
α5(IV)NC1'	Wieslab	MAB5	Monoclonal mouse IgG	1:75	aceton or PFA	IF	I
CD31	R&D Systems	AF806	Polyclonal sheep IgG	1:50	aceton or PFA	IF	I
Type I collagen	Abcam	Ab 34710	Polyclonal rabbit IgG	1:1200	-	IHC	II, IV
Type IV collagen	Fischer Scientific	Mp Cappel	Polyclonal rabbit IgG	1:75	-	IHC	II, IV
Type IV collagen	Chemicon	AB748	Polyclonal rabbit IgG	1:40	PFA	IF	IV

Antigen	Company	Code	Type	Dilution	Fluorophore	Application	Paper
Secondary antibodies							
Sheep IgG	Jackson Immunoresearch	713-096-147	Donkey	1:100	FITC	IF	I
Mouse IgG	Jackson ImmunoResearch	715-026-150	Donkey	1:100	TRITC	IF	I
Rabbit IgG	Jackson ImmunoResearch	711-025-152	Donkey	1:100	TRITC	IF	IV

Table 2. *Primary and secondary antibodies used.*
IF=Immunoflourescence, ICH= Immunohistochemistry, TRITC= Tetramethyl rhodamine isothiocyanate, FITC= Flourescin Isothiocyanate, PFA= Paraformaldehyde.

Chapter 11: Classification of metastatic growth patterns of CLM

In paper II the metastatic growth pattern of CLM was classified based on Vermeulens original description[92]. This meant that the dominant pattern, present in the largest CLM with the largest available border between tumour and parenchyma was used for classification. The growth pattern was considered dominant if one growth pattern represented more than 50 % of the tumorous border. A H&E stain and a Reticular stain was used for determining the type of growth pattern. In paper III we modified this classification by the addition of the CLM type ”mixed”. This classification was applied when there was no dominant growth pattern present.

Chapter 12: Cell lines and culturing

Experiments on the well-established, commercially available colorectal cell lines HT-29 (ATCC, HTB-38), LoVo (ATCC, CCL-229), SW-480 (ATCC, CCL-228) and SW-620 (ATCC, CCL-227) was preformed in paper IV. In this paper the type IV collagen production in these cell lines was investigated and the cell lines were used in the development of a novel organotypic liver metastatic model.

HT-29 is a cell line of human colon cancer that has been extensively studied and was isolated in 1964 from a female 44-year old patient. LoVo is a well characterized cell line isolated from a lymph node metastasis of the supraclavicular fossa in 1971 of a 56-year old male patient with CRC (Duke´s C). SW-480 is a cell line isolated from the primary tumour in a 50 year old male patient with colon cancer (Duke´s B) and SW-620 was one year later isolated from a lymph node metastasis in the same patient.

HT-29 was cultured according to recommendations with ATCC-formulated McCoy´s 5a Medium Modified (No 30-2007) and LoVo with ATCC Kaighn´s modification of Ham´s F-12 medium (F-K12 Medium) (No 30-2004). The medias was supplemented with 10% fetal calf serum (FCS), penicillin (10^5 IU/L) and streptomycin (100 mg/L) and incubated in 37°C with an atmosphere of 5% CO_2. SW-480 and SW-620 was cultured according to the manufacturers recommendations with ATCC-formulated Leibovitz's L-15 Medium (No. 30-2008) supplemented with 10% fetal calf serum (FCS), penicillin (10^5 IU/L) and streptomycin (100 mg/L) and incubated in 37°C in normal atmosphere.

Chapter 13. The organotypic liver metastatic model

A new organotypic model for cancer invasion using human uterine leiomyoma tissue as an authentic stromal context was recently established [133]. In paper IV we further developed this model into a novel organotypic

liver metastatic model based on human liver tissue, in order to investigate if and how cancer cells can invade and react to an authentic human liver stroma devoid of other cells, and if this stromal context can elicit a stromal response by the CRC cells.

Human liver tissue was collected during surgery from patients that underwent liver resection due to malignant or benign liver lesions (n=6). Due to freezing, there were no remnant live cells in the liver tissue. Each set of experiment used liver tissue from the same patient. At the start of the experiment the liver tissue was semi-thawed and cylinders of tissue were retrieved using an eight mm biopsy punch. A scalpel was used to cut the cylinder into a height of 5 mm. The liver cylinders were incubated in cell media for 48-36 hours in +4°C to rinse the tissue from toxins. Cell media for the intended CRC cell line was used for this purpose.

At the day of cell incubation of the tissue the liver cylinders were equilibrated in room temperature for two hours. The liver cylinders were placed in transwell chambers with a semi-permeable membrane (diameter 6.5 mm, Corning Inc., Corning, NY, USA). $5x10^5$ cells suspended in 50µL of cell media were placed on top of the liver cylinder in the transwells. The lower chambers of the transwells were filled with 1 mL of media, allowing for nutrition. The cells were allowed to attach to the liver over-night in an incubator (**Figure 10**). The following day the cylinders with CRC cells were removed from the transwells, and transferred onto a nylon membrane placed on top of steel grids in 12-well plates filled with 1-3 mL of media (**Figure 10**). The liver cylinders were incubated for up to 15 days, and media was changed and collected every second to third day. Termination of the experiment was done day five, 10 and 15. At this time the liver cylinders were placed in in paraformaldehyde for fixation and later paraffinated to allow for immunohistochemical analysis.

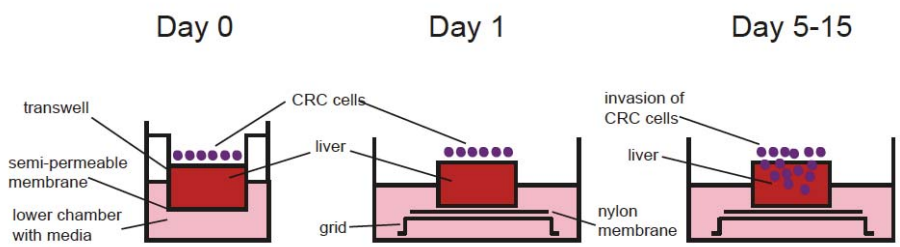

***Figure 10**- The organotypic liver metastatic model. The addition of CRC cells at day 0, the transfer of the liver with the attached cells from the transwell to the metallic grid at day 1 and the CRC cell invasion at day 5-15.*

Chapter 14: Statistics

Statistical analysis was preformed using software SPSS version 17.0/19.0 and STATA version 11.

In paper I the circulating levels of type IV collagen were compared between CLM patients, CRC patients and controls. The samples were not following normal distribution, verified by a normality test. For this reason non-parametric tests were used as follows: statistical analysis comparing non-related samples was preformed using the Kruskal-Wallis test and to compare related samples in the CLM group Wilcoxon signed rank test was used.

In paper II differences between groups were analysed using odds ratio (OR) and the Pearson´s chi square test. To estimate differences between disease-free intervals the Mann-Whitney U-test was used. Probability of survival was estimated by using the Kaplan-Meier estimator, and differences were calculated using a log-rank test.

In paper III the data regarding tumour marker levels were assumed not to be normally distributed. The Mann-Whitney U-test was used to estimate differences between the groups. The inter-rater agreement regarding the classification of CLM growth pattern was calculated by using Cohen´s Kappa coefficient. The probability of CLM was calculated using a logistic regression model and the estimated probabilities were used in a ROC analysis to calculate the area under the curve (AUC) for different models. In the ROC analysis for the tumour markers every operation was included as an event, and the samples for the seven patients that underwent repeated liver resection were treated as independent observations. When estimating the prognostic value of the tumour markers in relation to survival, or the metastatic pattern of CLM in relation to survival, the start for the observation was set to the time of the first surgical intervention, whether patients underwent one or more than one liver resection. Differences in survival probability between groups were tested using the Log-Rank test.

Comparing circulating type IV collagen levels in paper IV between groups was preformed by the use of Kruskal-Wallis and the Mann-Whitney U-test, assuming that data was not following normal distribution. A two-tailed analysis was used. The level of significance was set to 5% for all tests.

IV. Results

Chapter 15. Circulating type IV collagen plasma levels

Circulating type IV collagen and CEA levels of the different groups compared are presented in **Table 3**. Values are presented as mean±SD unless otherwise specified. CEA is presented in median and range.

Table 3.

	Collagen IV (ng/mL)	**CEA** (ng/mL)	**Gender** (percentage F/M)	**Age** (mean, range or SD)
Paper I				
CLM Pre-sample (n=15)	384±244	206 [4.5-2155]	27/73	63[45-76]
CLM Post-sample (n=14)	296±169	-	-	-
CLM Progress-sample (n=12)	498±241	-	-	-
Controls (n=8)	106±35	-	62/38	51[27-71]
CRC (TNM I–III) (n=32)	118±37	-	34/66	66[26-80]
Paper III				
CLM Preoperative (n=94)	170.1±72.5	7.6 [0.8-2073]	33/67	63.5±8.6
CLM Postoperative	-	-	33/67	66.2±7.5
Recurrent CLM (n=14)	214.0±145.4	-	-	-
No recurrent disease (n=10)	82.4±13.7	-	-	-
Controls (n=118)	104.4±33.0	1.3 [0.2-4.2]	51/49	54.6±10.3
Paper IV				
Epithelial LM (n=23)	350.7±398	-	61/39	62.4[34-71]
Neuroendocrine LM (n=9)	245.6±223.6	-	11/89	62.7[46-68]
Other LM (n=4)	93.8±94.9	-	50/50	52.8[24-71]
Benign lesions (n=5)	123.3±46.4	-	40/60	60.3[49-75]

15.1 Elevated circulating type IV collagen in patients with CLM

In paper I plasma samples (referred to as ”presample”) from patients with non-resectable CLM treated with chemotherapy was measured and found to be significantly higher (n=15, 384±244 ng/mL, p<0.001) than preoperative samples from healthy control patients undergoing surgery for benign disease (n=8, 106±35 ng/mL) and preoperative samples from patients with primary CRC (TNM stage I-III) (n=32, 118±37 ng/mL) (Paper I, Figure 2A). There were no significant differences in type IV collagen levels in different stages of the primary tumour (TNM stage I-III/ Duke´s A-C) (Paper I Figure 2B).

During palliative treatment with either intra-arterial or intra-portal regime the circulating levels of type IV collagen decreased, but not significantly (n=14, 296±169 ng/mL) and then increased at the time of progress (n=12, 498±241) when compared to the presample and postsample retrieved during 2-4 months of treatment (p<0.01 and p<0.01, respectively) (Paper I, Figure 2 A).

The circulating type IV collagen levels were also measured in preoperative samples from patients with resectable CLM (n=94) and compared to a control group of healthy individuals (n=118) with the preoperative samples of CLM being significantly higher (170.2±72.5 ng/mL) than levels in the controls (104.3±33 ng/ml, p=0.001) (Paper III, Figure 2A). The plasma levels for the control groups were within the range stated as normal by the ELISA manufacturer.

These findings taken together suggest that circulating type IV collagen is a promising candidate tumour marker for CLM.

15.2 Circulating type IV collagen levels can detect CLM recurrence

For 27 patients out of the 94 in paper III with a preoperative sample analysed there was also a postoperative sample available (collected 6-42 months after surgery). The levels in this group were analysed and correlated to whether they had recurrent disease or not at the time of the collected sample. 17 patients had recurrent disease and presented with significantly elevated levels of type IV collagen (200.1±136.9 ng/mL) compared to the group with no recurrence (n=10, 82.3±13.7 ng/mL, p=0.001). 14 of the 17 patients had recurrent CLM with increased type IV collagen levels (214±145 ng/mL) compared to the disease-free group (n=10, 82.3±13.7 ng/ml, p=0.001). Three patients in the recurrent group had non-hepatic spread of the disease.

The results support that circulating type IV collagen levels can be used to monitor resected CLM patients and detect CLM recurrence.

15.3 Circulating type IV collagen levels reflects the hepatic tumour burden

In paper I we found that the circulating levels of type IV correlated to the hepatic tumour burden of CLM, based on estimations on MRI and CT scans prior to the start of oncological treatment (Paper I, Figure 3).). The patients were grouped into CLM with <25% affected liver parenchyma (n=2) with an average type IV collagen level of 137±3 ng/mL, CLM with 25-50% affected liver parenchyma (n=10) and a type IV collagen level of 316±175 ng/mL and finally CLM with >50% of the liver parenchyma affected (n=3) with an average type IV collagen level of 753±104 ng/mL. The difference in circulating collagen IV between the groups was significant ($p<0.01$).

As earlier mentioned in chapter 13.1, the levels decreased during oncological treatment and increased at the time of disease progress (Paper I, Figure 2A). Taken together, these results supports that the levels of circulating type IV collagen reflects the hepatic tumour burden and can be used as a monitoring marker to evaluate the tumour response of oncological treatment in CLM patients.

15.4 Comparison of CEA and type IV collagen as tumour markers for CLM

In paper I circulating type IV collagen was found to be elevated in 100% of the patients (n=12) and the conventional marker CEA elevated in only 60% of the patients (n=10) (Paper I, Figure 4). The collagen IV levels decreased during treatment and increased at disease progression. These results imply that type IV collagen could be better than CEA as a tumour marker in CLM. However, the group were to small to allow for statistical comparison.

For this reason we compared circulating collagen IV and CEA in the 94 CLM patients in paper III. Here a ROC analysis of resectable CLM patients with preoperative samples were compared to healthy controls in a joined ROC analysis, revealing that circulating type IV collagen was not better than CEA alone. However, the clinically used cut-off for CEA is 5 ng/mL and the optimal cut-off based on our material in the ROC analysis was 2.6 ng/mL. When using the clinically relevant cut-off of 5 ng/mL and the calculated cut-off for collagen IV of 115 ng/mL, merely 49% of the patients (n=44) presented with elevated CEA levels compared to 81% of CLM patients having elevated type IV collagen levels (n=76).

When combining the two markers in a joined analysis we found that the combination was significantly better in detecting CLM than CEA or type IV collagen alone ($p=0.001$ and $p<0.001$, respectively) (Paper III, Figure 3B). These results shows that a combination of the conventional marker CEA and circulating type IV collagen is superior than any of the markers alone and that when using the clinically recommended cut-off for CEA, type IV collagen is more sensitive in detecting CLM than CEA.

15.5 Circulating levels of type IV collagen and CEA as prognostic factors

An analysis with a combination of circulating type IV collagen and CEA was performed to discover whether the tumour marker levels correlated to survival, and therefore could have prognostic relevance. In this analysis patients were grouped based on both markers being below the median value, one marker above median value or both markers above the median value (Paper III, Figure 5A).

The analysis revealed that patients with low levels of both markers (n=18) had the best survival (89%), patients with one marker above median (n=43) displayed a survival of 70% and patients with both markers elevated (n=17) had the poorest survival of 47% (Paper III, Figure 5A). In conclusion, this reveals that the levels of type IV collagen as well as CEA is correlated to survival and provides prognostic information.

15.6 Increased circulating type IV collagen levels in LM of epithelial origin

In paper IV preoperative plasma samples from patients that underwent liver resection due to liver metastases (LM) of different embryological origin (n=41) (**Table 3** and Paper IV, Table 2) were analysed, and compared with preoperative plasma samples of patients that underwent surgery for benign liver lesions (n=5). The group with benign liver lesions underwent liver resection due to suspected malignant disease. Patients with LM of epithelial origin (n=23) presented with significantly higher levels (350.7±398 ng/mL) of circulating type IV collagen than patients with LM of other embryological origin (n=4) (93.8±94.9 ng/mL) and benign liver lesions (n=5) (123.3±46.4 ng/mL) (p=0.002 and p=0.009, respectively). There were no significant difference between the circulating levels in patients with LM of epithelial origin (n=23) and LM of neuroendocrine tumours (n=9) (245.6±223.6 ng/mL)(p=0.414).

These findings reveal that LM of epithelial and some LM of neuroendocrine origin produces high circulating levels of type IV collagen.

Chapter 16. Expression patterns of stromal collagens in normal colorectum, normal liver, CRC, CLM and in other LM

The expression patterns and distribution of stromal collagens were analysed and classified in normal colorectal and liver tissue as well as in CRC, CLM and LM of other origin than CRC. In tissue from CRC the most invasive part of the tumour was retrieved for analysis, and in CLM the largest metastasis was used for the tissue analysis.

16.1 Expression patterns of a α-chains of type IV collagen in normal liver and in CLM

The different α-chains of type IV collagen was investigated in normal liver and in tissue of CLM (Paper I). In normal liver tissue the α1(IV)- and α2(IV)-chains was expressed in the space of Disse/ the perisinusoidal space and in vascular BMs. There was no expression of the α3(IV)- and α5(IV)-chains in normal liver or in CLM tissue. In tissue from CLM the α1(IV)- and α2(IV)-chains were strongly expressed in the DR of the tumour as well as in vascular BMs (Paper I, Figure 1). There are no specific antibodies for the α4(IV)- and α6(IV)-chains commercially available, therefore expression of these chains could not be studied. These findings display a strong expression within the DR of CLM.

16.2 Stromal collagen composition in matched CRC and CLM tissue

16.2.1 Stromal collagen composition in primary CRC and normal colorectum

In paper II the stromal expression and composition of type I, III and IV collagen as well as as reticular fibers in patients with primary CRC and the subsequent CLM arising from that primary tumour. This group was compared to primary CRC that never metastasized (for a follow-up time of five years). The expression of collagens and reticular fibers was also studied in normal colorectal tissue. The expression of type I collagen in normal tissue was predominantly located in the interstitial compartment, and type IV collagen in the BM and in vascular BM (Paper II, figure 1). The expression of collagens was graded in the DR and in the vicinity of the cancer cells. Liver-metastatic CRC displayed significantly higher expression of type I collagen in the DR (p=0.002) (Paper II, Figure 1) and type IV collagen was expressed intensely in the vicinity of the cancer cells in the liver-metastatic CRC when compared to non-metastatic CRC (p=0.004) (Paper II, Figures 1 and 2), but expressed only at low levels in the DR in both the non-metastatic and in the liver-metastatic CRC.

Patients that had received preoperative radiotherapy for rectal cancer displayed a higher collagen expression in general in both the control group (n=9) and in the CLM group (n=10). The expression of type I collagen in the DR of primary CRC, and the expression of type IV collagen in the vicinity of the cancer cells correlated to a high differentiation of the CRC (p=0.019 and p=0.052, respectively) (Table II). There was no correlation of stromal composition of collagens and the T stage. Type III collagen was only a minor part of the stromal collagens observed in CRC. These findings in primary CRC were correlated to the risk of developing a subsequent CLM, as presented in chapter 17.3 Thus, the results indicate that there is a difference in stromal collagen composition between the liver-metastatic CRC and the non-metastatic CRC.

16.2.2 Stromal collagen composition in CLM and normal liver

The metastatic growth patterns in CLM with matched primary CRC tumours (described in Chapter 15) were graded and related to collagen I, III and IV expression in paper II. In normal liver type I collagen is predominantly expressed in the portal tracts, type III collagen mainly within liver sinusoids (data not shown) and type IV collagen in vascular BM and in the liver sinusoids (Paper II, Figure 3). The *desmoplastic* type of CLM presented with a high type I and IV collagen expression both within the tumour and in the surrounding DR. The *pushing* type of CLM also had intense expression of type I and IV collagen within the tumour, but significantly lower expression of type I and IV collagen in the thin rim separating tumour cells from normal liver ($p<0.001$ and $p<0.001$, respectively). Type III collagen was only a minor component of the collagens studied in CLM (data not shown).

16.3 Correlation between stromal collagen composition in primary CRC and the risk of a subsequent CLM

In paper II we investigated the correlation between collagen composition in primary CRC and the risk of a subsequent CLM. High type I collagen expression levels in the vicinity of the cancer cells and in the DR correlated to an increased risk of being diagnosed with a subsequent CLM (OR=3.616, [95%] CI=1.604-12.286, and OR=6.00 [95%] CI=2.096-17.173, respectively). High expression of reticular fibers was also associated with an increased risk of being diagnosed with CLM (OR=3.419, [95%] CI=1.161-10.123). However, the highest risk of developing a subsequent CLM was found in patients with strong type IV collagen expression in the immediate vicinity of the CRC cells (OR=11.3, [95%] CI=3.706-34.726).

These results indicate that the risk of developing a CLM is related to the stromal collagen composition of the primary tumour.

16.4 Type IV collagen expression in LM of other origin than CRC

Due to our previous findings in CLM we also wanted to study the tissue expression of type IV collagen in LM other than CRC. In paper IV, moderate to intense levels of type IV collagen expression was found in LM of epithelial origin (n=17), while neuroendocrine LM (n=8) varied in their collagen IV expression from low to intense expression (Paper IV, Figure 1). The circulating type IV collagen levels in neuroendocrine LM correlated with the tissue tumour expression. Patients with LM of other embryological origin expressed low levels of type IV collagen (n=5) (Paper IV, Figure 1), except for one patient with a leiomyosarcoma where there was an intense type IV collagen staining. Patients with benign liver lesions (n=4) expressed low to

moderate type IV collagen in the tissue analysed (Paper IV, Figure I), except for one patient with a large cavernous hemangioma engaging the right liver lobe where both the tissue expression was very strong as well as the circulating type IV collagen levels high (201 ng/mL).

These findings indicate that the up-regulation of type IV collagen in LM might correlate to the embryological origin of the tumour, with epithelial and some neuroendocrine tumours presenting with a high tissue expression, compared to LM of other embryological origin.

Chapter 17. Metastatic growth patterns of CLM

Different metastatic growth patterns have earlier been presented by Vermeulen et al. [92]. In paper II we verified the earlier described metastatic types of 48 patients with CLM as follows (Paper II, Table 1): The *desmoplastic* type of CLM (47 %, n=22) in which there is a pronounced DR separating the liver parenchyma from cancer cells with a rich inflammatory infiltrate present. The *pushing* type of CLM (53 %, n=25) is characterized by the cancer cells pushing the normal liver parenchyma away with little or no DR present and only little inflammation present. The third type called the *replacement* growth pattern in which CRC cells grow within the normal sinusoidal architecture of the liver could not be found within patients in paper II. One patient in this paper was excluded from the tissue analysis due to a non-representative specimen of the CLM that could not be used for classification.

In paper III we analysed the growth patterns in a larger cohort and modified the classifying system as previously described in material and method, with a new group called *mixed* where more than one growth pattern was present and none dominated. Two independent observers (HN and MB) classified the metastatic growth pattern of the CLM, with an inter-rater agreement of 0.86. In this cohort CLM growth patterns in 67 patients were classified. 54% (n=36) of the patients had a *pushing* type of CLM, 30% (n=20) were classified as *desmoplastic*, 3% as *replacement* (n=2) and 13% (n=9) as *mixed*. For patients that underwent repeated liver resections (paper II and III) the metastatic growth pattern was always the same, indicating that this trait might be constant and related to tumour biology and/or host response. In paper I also patients that had undergone chemotherapy with a significant tumour regression were classified (n=5). In paper III all patients with substantial tumour regression due to chemotherapy in the tissue analysed was excluded, to eliminate possible faulty classifications due to treatment. Furthermore, we also analysed the metastatic growth patterns of CLM in patients that had not received any chemotherapy at least 3 months prior surgery (n=28). These results support that chemotherapy does not affect the metastatic growth pattern, but that a pronounced regression makes it hard to classify the type of CLM.

We also analysed in the matched primary CRC and CLM cohort, whether stromal findings in the primary CRC could be related to the metastatic type of CLM, and found that there was no correlation in stromal composition of primary CRC and the metastatic type of subsequent CLM.

17.1 Metastatic growth pattern related to survival

To clarify whether the growth pattern of CLM has an impact of clinical outcome, a survival analysis was preformed (Paper II and III). This revealed that patients with the *pushing* type of CLM (n=25) display a significantly poorer survival, when compared to patients with the *desmoplastic* type (n=22) (Paper II, figure 4 and Paper III figure 5B). For patients in the *pushing* group the mean survival was 63±6.9 months, compared to 93±10.5 months in the *desmoplastic* group (Paper II). There was a significant difference in survival at 60 months, with the only long-term survivors found in the *desmoplastic* group (p=0.046, Paper II, figure 4). Additionally, the cause of death was characterized for the patients in paper II. This revealed that patients with the *pushing* type of CLM developed a more disseminated disease than patients with the *desmoplastic* growth pattern (Paper II, table III). Nine of the patients in the *pushing* group developed lung metastases compared to none in the *desmoplastic group*. Furthermore, patients with the *desmoplastic* type of CLM mainly had disease recurrence restricted only to the liver.

In paper III we wanted to verify our findings and performed a survival analysis of the patients with a *pushing* versus a *desmoplastic* type of CLM. This analysis revealed that also in this cohort, patients with the *pushing* type of CLM (n=32) had a poorer outcome than the *desmoplastic* group (n=18) with a mortality of 44% (n=14) in the *pushing* group compared to 11% (n=2) in the *desmoplastic* group (p=0.011). The other groups (*mixed* n=9 and *replacement* n=2) were to few to allow for statistical analysis.

These findings reveals that the metastatic growth pattern in CLM has clinical importance and is related to survival, with the *pushing* type of CLM clearly representing a more aggressive disease.

17.2 Relation between metastatic growth pattern and circulating type IV collagen levels

In paper III the circulating type IV collagen levels were correlated to the metastatic growth pattern of CLM. There was no relationship between the major types of CLM (*desmoplastic* and *pushing*) and the circulating collagen IV levels. The levels of circulating type IV collagen for each metastatic group were: *pushing* 162.9±75 ng/ml (n=36), *desmoplastic* 169±56 ng/ml (n=20), *mixed* 156±52 ng/ml (n=9) and *replacement* 101±46 ng/ml (n=2).

As previously described the metastatic growth pattern was also classified in a subcohort of 28 patients that had not received any chemotherapy prior liver resection (Paper III, Table 1). In this smaller cohort the circulating levels of type IV collagen were 158.1±54.0 ng/ml (n=8) in the *desmoplastic* group, 144.7±74.3 ng/ml (n=15) in the *pushing* group, 113.8±24.8 ng/ml in the *mixed* group (n=4) and 110.7 ng/ml in the *replacement* type of CLM (n=1). There was no significant difference in type IV collagen levels between the *pushing* and the *desmoplastic* group in *subcohort II*. The number of patients within the *replacement* and the *mixed*

group were too small in both the main cohort of 67 patients, as well as in the subcohort of 28 patients to allow for statistical comparison.

There were no significant differences in the type IV collagens levels between *desmoplastic* and *pushing* type of CLM, regardless of whether they hade received chemotherapy prior to surgery or not (p=0.632 and p=0.144, respectively). These results reveal that the metastatic type of CLM is not related to circulating type IV collagen levels.

17.3 Disease-free interval in the metastatic types of CLM

In paper II the desmoplastic type of CLM (median 0 [range 0-41 months]) was associated with a shorter disease-free interval between diagnosis of the primary CRC and CLM, when compared to the pushing type (median 7 [range 0-52 months]) (p=0.047). However, these findings were not verified in the larger cohort, where the difference in disease-free interval between the groups was not significant (p=0.09).

Chapter 18. A novel organotypic liver metastatic model

The CRC cell lines HT-29 and LoVo were grown on plastic slides, where both cell lines endogenously produced type IV collagen as verified by immunofluorescence (Paper IV, Figure 2). The cell lines SW-480 and SW-620 did not produce any type IV collagen (data not shown), and did not survive or invade into the liver model. Both cell lines HT-29 and LoVo did invade the liver (Paper IV, Figure 3). HT-29 invaded the liver more extensively than LoVo (Paper IV, Figure 3A and C), but none of the cell lines produced a stromal reaction in the organotypic liver metastatic model.

Both HT-29 and LoVo initiated the invasion of the liver at stroma- and type IV collagen rich structures such as the vascular BM (Paper IV, Figure 4). From this initiating point the CRC cells attached and grew into the liver, enhancing the cellular invasion over time (Paper IV, Figure 5). There were no other viable cells found in the collected human liver. The results show that human liver can be used as an organotypic model to study the invasion of CRC cells. In addition it was shown that a normal liver stroma, devoid of living stromal cells, with invasion of CRC cells are not sufficient to induce a stromal response.

V. Discussion

Chapter 19. Expression patterns of stromal collagens in CRC and CLM

Expression of type IV collagen in normal colorectum is restricted to the BM and vascular BM. Normal distribution of the α(IV)-chains has earlier been described, with the finding of the α1-, α2-, α5-, and α6-chains in the BM of the mucosal epithelium and the gland crypts, and the α3- and α4 – chains in BMs of the luminal surface epithelium [76]. The definition of an invasive cancer is in fact the degradation of the normal BM, where the cancer cells starts to invade the underlying tissue [78-80]. Type IV collagen in the most abundant BM protein. In primary CRC this process is characterized by the loss of the structural BM integrity [76] and the cause of type IV collagen degradation is related to the activity of MMPs in the tumour microenvironment [77].

We have in paper II seen that the normal type IV collagen rich BM is lost in CRC, and that type IV collagen becomes a component of the desmoplasia in CRC. We also observe a difference in type IV collagen expression and composition between non-metastatic CRC and liver-metastatic-CRC. CRC that is liver-metastatic shows intense staining for type IV collagen in the direct vicinity of the cancer cells at the invasive front of the tumour. This was strongly correlated to the risk of being diagnosed with a subsequent CLM. The possible mechanisms of this are further discussed in chapter 22. We also found that the expression of type IV collagen in the vicinity of the CRC cell correlated to tumour differentiation. Oka et al have demonstrated that a loss of type IV collagen staining (α1 and α2-chain) in primary CRC is related to poorer differentiation of the tumour [76].

Type IV collagen in normal liver is expressed in the liver sinusoids and in vascular BM. We showed that in CLM the architecture of the liver is interrupted, and that in CLM an intense expression of type IV collagen is observed, with type IV collagen constituted by the α1α1α2 protomer. This increased expression in CLM is supported by the same findings in other studies [85,111]. Furthermore, we investigated type IV collagen expression in the different metastatic types of CLM, with the finding that the desmoplastic type of CLM differed significantly with a high expression of type IV collagen in the surrounding DR of the tumour, when compared to the pushing type of CLM. This could mainly be due to the fact that the pushing type of CLM lacks a DR separating tumour from parenchyma. Both growth patterns of CLM had high type IV collagen expression within the tumour.

Type I and III collagen are the major collagens of the interstitial compartment in the colorectum [75]. We found that type III collagen was a minor component of the DR in CRC and that type I collagen was the major component. Both collagens were expressed in the same areas of desmoplastic stroma of the tumour. Type I collagen expression in the DR of the tumour was higher in liver-metastatic CRC compared to the non-metastatic control

group and also correlated to a high risk of subsequent CLM diagnosis, although with a lower OR than what was observed for type IV collagen.

Type I and III collagen was also graded in the corresponding CLM, with the findings that the desmoplastic type of CLM expressed high levels of type I and III collagen in the DR surrounding the metastasis, in contrast to the pushing type. As for type IV collagen, type I collagen was expressed intensely within the tumour. Type III collagen was also expressed within the tumour, but to a lesser degree than type I and IV collagen. Conti presented in an article that type I collagen was the major collagen found in CLM, and that type IV collagen was not quite as highly expressed [85]. Based on our work we cannot agree, since both collagens are major parts of the DR seen in CLM. Furthermore, to make such a comparison more appropriate methods such as double staining should be used.

The expression pattern and distribution of the stromal type I, III and IV collagen change in CRC, with type IV collagen in CRC being expressed close to cancer cells in the liver-metastatic CRC cells and type I and III collagen being mainly found in the general DR. The same distribution is however not observed in CLM, where all three collagens are found in the DR of the tumour. This might be the reason to why patients with CLM, but not patients with CRC presents with high circulating type IV collagen levels.

Chapter 20. Circulating type IV collagen as a tumour marker for CLM

Ambiru et al reported in 1994 that patients with CLM display increased circulating levels of type IV collagen [111]. To our knowledge no other studies in this matter have been published. We have in paper I and III shown that circulating type IV collagen is a promising new tumour marker for CLM. In paper I the circulating levels of type IV collagen in non-resectable CLM are very high, compared to healthy controls and to primary CRC (TNM stage I-III), and that the levels seem to correlate to the hepatic tumour burden. Furthermore, we show that this marker decreases in response to chemotherapy and increases at progression of the disease. This means that type IV collagen could potentially be used to monitor response to oncological treatment as a monitoring marker.

The exact mechanism to why increased type IV collagen levels are found in CLM patients is not known. We have in paper I and II shown that CLM has a high expression of type IV collagen within the tumour. This could be due to increased breakdown in the tissue, but also due to an increased production in the tumour. In paper III we established that the circulating levels of type IV collagen do not have a relation to the metastatic growth patterns of CLM. Despite that there was a difference in type IV collagen within the surrounding DR of the CLM, expression inside the tumour was generally high and this is probably the reason to why no difference is seen. In paper IV we discovered that LM of epithelial origin, but also some LM of neuroendocrine origin present with high circulating type IV collagen levels, and that these levels correlate to the tissue expression of the LM. This supports the idea that epithelial cells metastasizing to the liver evokes an

increase in circulating levels of type IV collagen, much in the same manner as CLM does.

Circulating type IV collagen has been presented as a marker for hepatic fibrosis, but is not used in clinical practice. The hepatic wound healing response is characterized by an increased deposition of ECM proteins such as collagens and can be evoked by many different stimuli [134]. The desmoplastic stromal reaction observed in CLM is likely a result of the combination of the biological properties of the CRC cells and the host response. The HSC is a key-player in the hepatic wound response and might also be central in the desmoplastic reaction of CLM.

We have in paper I and III compared type IV collagen to the conventional tumour marker CEA, where the combination of the to markers in a joined ROC analysis revealed that the combination is superior to either CEA or collagen IV alone. As earlier discussed, type IV collagen is not better than CEA in the ROC-analysis based on our material in paper III. However, this is explained by the fact that the cut-off in the ROC analysis was calculated to 2.6 ng/mL compared to the clinical cut-off of 5 ng/mL. When using the clinical cut-off level, a much larger percentage of patients with CLM (81%) had increased type IV collagen levels, when compared to CEA levels (49%). Circulating type IV collagen is a stable and easy protein to detect in blood samples, and the optimal cut-off based on paper III is calculated to 115 ng/mL. CEA has its major role as a surveillance marker for CLM, but 50% of patients do not show increased levels [106] [107] and these findings were verified in paper III.

In this thesis it is shown that circulating type IV collagen is a good candidate marker for the diagnosis of CLM, evaluation of non-resectable CLM patients undergoing oncological treatment and monitoring of resected CLM patients to find recurrent disease. Furthermore, we have shown that the combination of CEA and type IV collagen is superior to one marker alone. Type IV collagen and CEA are markers that reflects different compartments of the tumour biology with CEA as a marker of the cancer cells and type IV collagen of the stromal compartment.

Chapter 21. The role of type IV collagen in the liver metastatic process

To our knowledge no one has earlier studied the composition of collagens in matched samples of primary CRC and the subsequent CLM, and compared this to non-metastatic primary CRC (paper II). Here we found that in liver metastatic CRC, both type I and IV collagen expression correlated to a higher risk of developing a CLM. The highest risk for a subsequent CLM was correlated to intense type IV collagen expression in the vicinity of the cancer cells of the primary tumour.

Although it is not established which cells are responsible for this production it is highly likely that the CRC cancer cells might be their own ECM producers and/or affecting stromal cells such as fibroblasts to initiate such a response. Furthermore, we showed that the two CRC cell lines HT-29 and LoVo both produces their own type IV collagen, and that these cell-lines

could establish an invasion in the organotypic liver metastatic model. Two other cell lines (SW-480 and SW-620) were not found produce type IV collagen, and these cell lines did not invade the liver model. It has been shown earlier that a murine lung carcinoma cell line over-expressing type IV collagen generated a highly liver metastatic cell line, and that this was due to escape of cell aniokis in a collagen IV/ α2 integrin/ FAK dependent manner and also an increased response to IGF-1 [91]. Furthermore, when type IV collagen in the same cell line was blocked, the liver metastatic potential was highly reduced [91]. Yoshimura and colleagues also showed that a liver metastatic B16 melanoma cell line differed in the composition of adhesion molecules, compared to non-liver-metastatic cell lines, where there was a distinct over-expression of the integrin α2-subunit [135]. Furthermore, this liver-metastatic preference was lost when α2 integrin was blocked. The α2 integrin mediated binding to type IV collagen has also been established in activating focal adhesion kinase, a process important for the formation of metastasis [135]. Type IV collagen is highly exposed in the liver sinusoids and these are directly exposed to the portal blood from the intestine, which is the common haematogenous route for CRC cancer cells to spread to the liver. Since there is evidence pointing to that CRC cells prefer to anchor through integrins to type IV collagen one could state that the liver is a very permissive soil for these cells to establish a growth within.

We have shown in paper IV that not only CLM, but also other LM of epithelial origin present with an increased tissue expression and elevated circulating levels of type IV collagen. All normal epithelial cells need contact to a BM and type IV collagen is the major BM protein. It has been shown that when an epithelial cell looses BM contact, apoptosis is initiated [136,137]. The interaction between type IV collagen and epithelial cells are mainly due to integrins (α1- and α2-subunits with the β1- subunit) [55]. Thus, there could be a biological survival benefit for cancer cells to be able to produce and/or initiate stromal cells to produce a BM-resembling stroma, providing the cancer cells with important epitopes for survival, proliferation and evading apoptosis.

These findings all indicate that type IV collagen might be of importance in the liver metastatic process of cancer cells. This could possible be used as a prognostic tool for surveillance of such CRC patients with a high risk of developing CLM and possibly also be used to identify molecular targets responsible for the liver-metastatic process.

Chapter 22. The clinical and tumour biological aspects of metastatic growth patterns

Undoubtly, the growth patterns of CLM have a major impact on patient outcome after liver resection. Patients with the pushing type of CLM displayed a significantly poorer survival and a disseminated disease, contrasting the desmoplastic type of CLM where the only long-term survivors were found.

We classified the metastatic patterns according to an earlier description of Vermeulen in 2001 [92] and modified this classification by

adding a fourth group, the mixed type, where no dominant growth pattern could be determined. In paper II and III there was in one dominant growth pattern present for the majority of the patients (100% and 87%, respectively). In paper II the proportion of the different CLM types resembled the proportions described in 2001 (**Table 4.**), with the exception that no CLM with a dominant replacement growth pattern could be found. The same group further, in relation to angiogenesis published a paper addressing the growth patterns of CLM in 2012 [138], where also the addition of a mixed group was introduced. In this work the authors modified the criteria for classification where a growth pattern dominating 75% of the tumour-parenchyma border was needed, to be considered as dominant. This paper showed a marked rise in the number of patients with a replacement type. However it was noticed that the pushing and the replacement growth patterns usually co-existed in the same CLM. We have noticed the same finding in our works and this implies that these two CLM types probably co-exist, and maybe they should be classified as one pattern. This study also revealed that patients with the pushing type had a poorer survival compared to the other groups [138]. The ratios of growth patterns in these four studies are presented in **Table 4**.

The difference in growth pattern ratio between paper II and III could be explained by the addition of the mixed group, but also by the fact that patients with a complete tumour regression due to chemotherapy could not be classified (n=16) and were therefore excluded. Thus one could speculate that there might be more complete-responders in the desmoplastic group that could not be classified, compared to the pushing group. In patients with the pushing type of CLM that had received preoperative chemotherapy it was not uncommon to see a partial tumour response with necrosis, but dense microsatellites of pushing islands of tumour cells surrounding a partial necrotic CLM. This could indicate that the pushing type of CLM responds poorly to chemotherapy.

A study in 2004 revealed that patients with LM of breast cancer all grew with the replacement growth pattern, compared to only 30% of CLM patients [139]. This might indicate that the poor survival of patients undergoing surgery for LM of breast cancer could be explained by the growth pattern, since the LM of this cancer form rarely grows with a desmoplastic capsule. This also raises another important question. Is long-term survival in patients with resected CLM mainly due to effects on patient with the desmoplastic growth pattern? Our results imply that patients with a pushing type of CLM have a very poor survival despite oncological and surgical treatment, and they develop recurrent disease with extrahepatic spread after liver surgery. Most importantly, what are the mechanisms responsible for the growth patterns? This could be due to intrinsic tumour biology and/or a host response. Since the desmoplastic type of CLM in general presents with an inflammatory infiltrate not seen in the pushing type it suggests that the inflammatory response might be of importance. This could be the reason to why these CLM are capsulated by a collagen-rich matrix with the inflammatory process being the response that initiates the production of the matrix capsule. The question then remains, what evokes this inflammatory process and what components in the pushing type is responsible for the non-

inflammatory and non-capsulating matrix response? As earlier mentioned, it has been shown that the stromal context is of importance in a cancer, where a malignant stroma can induce malignancy in normal cells and a normalized stroma can de-malignify cancer cells [140-142]. Patients that underwent repeated liver resection due to CLM in paper II and III, in general displayed the same growth patterns, indicating that this might be constant and related to tumour biology and or host/response.

Since, the desmoplastic type of CLM presents with a stromal response and inflammation, one could speculate that this CLM is detected by the host and the capsule observed could be a result of this host response. This could mean that the pushing type of CLM has traits that help it to avoid host defence mechanisms and escape the immunological response. An avoidance of a host response could explain why the patients with a pushing CLM have such a poor survival and disseminated disease.

Another reason for the lower mortality could be due to that a capsulated CLM could be easier to detect in radiological examinations compared to the pushing or replacement type. In paper II the correlation between disease-free interval between primary tumour and CLM was shorter in the desmoplastic type (p=0.047) but in paper III this difference was not significant (p=0.09).

Table 4. *A comparison of the ratios of classified growth patterns in the different studies presented.*

Metastatic growth patterns of CLM	**Vermeulen 2001** (n=26)	**Nyström 2012** (n= 47)	**Nyström 2013** (n=67)	**Van der Eynden 2012** (n=205)
Desmoplastic	42% (n=11)	47% (n=22)	30% (n=20)	34.6% (n=71)
Pushing	46% (n=12)	53% (n=25)	54% (n=36)	15.6% (n=32)
Replacement	12% (n=3)	0%	3% (n=2)	27.8% (n=57)
Mixed	-	-	13% (n=9)	17.6% (n=36)
Not classifiable	-	-	-	4.4% (n=9)

In this thesis it is clearly established that the metastatic growth patterns of CLM has a clinical importance. Further studies in this aspect should be directed towards identifying the responsible processes and whether they are related to tumour biology and/or host response. The pushing type of CLM needs a different treatment strategy to address the poor outcome for these patients. Furthermore, to do this we would have to be able to classify the growth patterns prior liver resection.

Chapter 23. A novel organotypic liver metastatic model

In this thesis we have developed a new organotypic liver metastatic model. The purpose was to create a model with normal intact ECM of the liver to

enable the study of CRC cell invasion. We have in this thesis found that the stromal reaction in CLM is important, both in the aspects of the liver-metastatic process with a difference in collagen expression in non-metastatic CRC versus liver-metastatic CRC, as well as a source for tumour markers. Additionally, the metastatic growth pattern with its stromal composition and distribution is of high clinical relevance.

Cancer cell invasion in a stromal context is usually studied in *in vitro* models of type I collagen or Matrigel, sometimes with the addition of stromal cells such as fibroblasts. Nurmenniemi proposed in 2009 that human uterine leiomyoma could be used as a more authentic model for cancer cell invasion [133]. However, despite that many women have leiomyoma of the uterus, metastases in this region are never seen, indicating that in human biology, for unknown reasons this is not a preferred site of metastasis of cancer cells.

The liver, in contrast is a major site of metastasis for a majority of cancers. This is explained by the portal drainage of the gastrointestinal channel, where cancer cells are spread by the haematogenous route to the liver. However many other non-gastrointestinal cancers also spread to the liver. Stephen Paget already in 1889 stated that the site of metastasis is not only due to the seed (the cancer cell), but also by the properties of the receiving soil (the receiving organ) [143]. Thus, the portal metastatic route is not the only explanation for the liver being an organ with frequent metastatic disease. The molecular biologics of this organ (the soil) is undoubtly also of importance.

By the development of the organotypic liver model we wanted to study the invasion of CRC cells into normal ECM of human liver and evaluate if these components were sufficient to initiate a stromal desmoplastic response with collagen production. Based on our material and cell lines no such response was seen. These results suggest that other components such as fibroblasts and other stromal cells are needed for the DR seen in CLM.

The establishment of the organotypic liver metastatic model as a functional model for CRC invasion in authentic liver stroma, devoid of other cells enables the studying of which components and mechanisms are responsible for the desmoplastic reaction observed in human CLM.

VI. Conclusions

The over-all conclusions of this thesis is that circulating type IV collagen is a promising candidate tumour marker for CLM. Furthermore the stromal composition of the primary tumour seems to be related to the risk of being diagnosed with a subsequent CLM, and type IV collagen can be of importance for the process of liver metastasis. The different growth patterns of CLM have important clinical value, with the pushing type of CLM representing a very poor patient survival, despite surgery and chemotherapy. Finally, we have developed a novel organotypic liver metastatic model for CLM based on human liver.

More specifically the conclusions are as follows:

1. The α1(IV)- and α2(IV) chains are the major component of type IV collagen in normal liver and CLM.
2. Tissue expression of type IV collagen is up regulated in CLM.
3. Circulating type IV collagen is elevated in patients with CLM and in patients with LM of epithelial origin, compared to healthy controls and colorectal cancer (TNM stage I-III).
4. The circulating levels of type IV collagen reflect the hepatic tumour burden and oncological treatment of CLM results in decreased levels of type IV collagen. Circulating type IV collagen can be used to detect recurrent CLM after liver surgery.
5. Circulating type IV collagen in combination with CEA is superior in detecting CLM, than using the conventional marker CEA alone. High levels of both markers are related to a poor survival.
6. Liver-metastatic CRC differs in its stromal composition of collagens compared to non-metastatic CRC, where liver-metastatic CRC express intense type IV collagen staining in the vicinity of the cancer cells.
7. There are three metastatic growth patterns of CLM, namely the desmoplastic, pushing and replacement growth pattern, which differs in their stromal collagen expression of the tumorous border.
8. The pushing type of CLM is a more aggressive tumour with a higher mortality and a more disseminated disease compared to the desmoplastic type.
9. The stromal composition of the primary CRC is not related to the metastatic type of CLM
10. Circulating type IV collagen levels are not related to the metastatic type of CLM.
11. A novel organotypic liver metastatic model using human liver as an authentic stromal context can be used to study CLM *in vitro*.

12. The type IV collagen producing CRC cells HT-29 and LoVo can invade the organotypic liver model and the non-type IV collagen producing cell lines SW-480 and SW-620 cannot invade the model.
13. The combination of an authentic extracellular liver matrix and CRC cells is not sufficient to evoke a stromal response normal seen in CLM in the organotypic liver metastatic model.

VII. Future perspectives

Tumour markers for CLM

The optimal tumour marker has many criteria to fulfil. Since we now have established that the BM protein type IV collagen is a promising candidate marker for CLM it could be of interest to evaluate other stromal proteins for this purpose, such as the BM proteins laminin, which is the second largest component of the BM, and perlecan, which is a proteoglycan. Other candidates could be hyaluronan, a glycosaminoglycan that has been proposed as a marker for breast and prostate cancer.

With the current ELISA method we are measuring the collagenous and 7S domain of type IV collagen. Since there are different fragments of type IV collagen (arresten, canstatin, tumstatin and hexastatin) we do not know if we are measuring fragments or the whole molecule. Specific ELISAs developed for the different fragments would be helpful, but today there are no such ELISAs commercially available.

Expression patterns of stromal proteins

Similarly to broaden the search for other circulating levels of stromal components as described above, this should also be accompanied by tissue analysis for the same proteins. In situ hybridization, to establish which cells are responsible for the secretion of ECM proteins would also be of great value and should be preformed in CRC and CLM tissue.

The metastatic growth patterns of CLM

Since the metastatic growth patterns of CLM has such an important clinical significance further studies revealing the cause of these patterns are needed. Correlation to other important tumour biological processes such as K-RAS and B-raf mutations, relation to MSI and expression of adhesive molecules interacting with the stroma should be preformed.

Furthermore, the inflammatory response and the possibility of the different types of CLM, as a result of host defence mechanisms should be evaluated. Are there tumour biological explanations to why the pushing type can avoid host defence mechanisms or is this due to the host own immunological response? Is the survival for patients with the desmoplastic type of CLM due to the host response and the capsule observed a protection? If so, can we identify the key elements for this reaction and possible develop therapeutic agents that would help host response to detect the tumour?

Maybe we can predict, by analysing the primary tumour, which tumours will metastasize, and which primary tumours will result in a certain type of CLM. It would be highly appealing if the molecular pattern of a CRC could predict these matters and to even possibly identify molecular targets for treatment.

To make the metastatic growth pattern possible to use in clinical practice new methods to verify this prior liver surgery should be developed, since we clearly are not providing the patients with a pushing type of CLM an optimal treatment. Radiological and possibly functional imaging methods

should be developed and evaluated on patients undergoing surgery for their CLM.

An optimal in vitro model to study CLM

With the development of the organotypic liver metastatic model we hope to be able to study the process of CLM with a special focus on the stroma. The next step would be to introduce stromal cells into the model to establish which components that are required for the stromal response seen in CLM as well as to possibly stimulate the cells with growth factors such as bFGF, VEGF and others to induce such a response. Identifying key processes such as, what elicits the stromal reaction seen in the different metastatic types of CLM, are highly desirable, and could help understanding why patients with a pushing type of CLM has such poor prognosis. Are there tumour biological properties that explain this? Or is it due to host mechanisms and the pushing type of CLM ability to avoid host defence?

Follow-up studies

The promising results of circulating type IV collagen would benefit in further studies, both involving patients undergoing liver resection and establishing when the type IV collagen levels are normalized after surgery, monitoring patients treated surgically for their CRC to establish the monitoring value of detecting recurrent disease, evaluating patients undergoing palliative chemotherapy to see if the marker can tell which patients are responders, possibly replacing some of the radiological examinations being preformed for this purpose.

The combination of type IV collagen and CEA is superior in detecting CLM than either marker alone, and this should be further evaluated in a larger cohort. These two tumour markers reflect different tumour compartments and the combination of such tumour markers could more sensitive and specific in detecting tumorous disease.

Collection of research material

Patients with resectable CLM are only a smaller part of the patients with CRC that develops disseminated disease. It would be of scientific value and of patient benefit to also be able to include these patients to a higher degree in biobanks and other studies. In Sweden about 500 patients undergo liver resection for CLM yearly. A collaboration between surgical centres would undoubtly be beneficial for the study of CLM, providing with tissue and blood samples, as well as clinical data.

The patients with CLM are a heterogeneous group of patients, being subjects to many different treatment strategies of chemotherapy and surgical intervention. For example, the use and actual benefit of neoadjuvant treatment is debatable, but many patients are subjects to this. This makes research harder, since the numbers of patients are relatively few and their treatment is not homogeneous, making comparison between groups and comparison over time challenging. A more standardized way of CLM treatment could make this easier and studies could be done in a better way.

Furthermore, liver metastatic surgery is a rapidly expanding field and we will probably see liver resection of other metastases that CRC increasing. There is however no national registry in Sweden that today takes into account what kind of liver metastasis that is treated. To enable for research on liver metastases of different origin a better classification in the national registry should be developed.

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