Circulating and genetic factors in colorectal cancer

Potential factors for establishing prognosis?

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"Kommer tid, kommer råd"

Jon Slind

ABSTRACT

Colorectal cancer (CRC) is defined as a cancer appearing in the colon or in the rectum. In Sweden, ~ 6300 individuals were diagnosed with the disease in 2014 and ~ 2550 individuals diagnosed with CRC die each year due to their cancer. Surgery is the main treatment option of CRC and a survival rate of ~ 10 % is estimated if distant metastases have developed. It is therefore of importance to find factors that may be useful together with tumour, node, metastasis (TNM) stage to establish early CRC diagnosis, prognosis and follow-up of CRC patients. The aim of this thesis was to study the possible association of *CD93*, *PLA2G4C*, PDGF-D and inflammatory cytokines with CRC disease progression.

In a prospective study approach *CD93* and *PLA2G4C* single nucleotide polymorphisms (SNPs) were of potential importance in CRC prognosis.

The T/T genotype of *CD93* was associated with an increased *CD93* expression in CRC tissue. Further, CRC patients carrying this genotype were associated with disseminated CRC at diagnosis and a lower recurrence-free survival after surgery. The A allele of a SNP of *PLA2G4C* was a stronger predictor for CRC-specific mortality than the conventional risk factors used in the clinic for selection of TNM stage II patients for adjuvant treatment. This indicates that the T/T genotype of *CD93* and the A allele of *PLA2G4C* may be potential genetic factors related to disease severity and spread. Furthermore, they distinguish CRC patients that may benefit from a more comprehensive follow-up and adjuvant treatment.

To study the putative involvement of PDGF-D in CRC the effects of PDGF-D signalling was studied *in vitro*. PDGF-D signalling altered the expression of genes of importance in CRC carcinogenesis and proliferation which was blocked by imatinib, a tyrosine kinase inhibitor. This indicates that PDGF-D signalling may be an important pathway in CRC progression and a potential target in CRC treatment.

The analysis of various inflammatory cytokines in plasma at diagnosis showed an association between high levels and increased total- or CRC-specific mortality two years after surgery. High levels of CCL1 and CCL24 was the only cytokines strongly correlated with a worse CRC prognosis after statistical adjustments and may be of interest for further evaluation.

In conclusion, this thesis presents circulating and genetic factors such as CD93, PLA2G4C, PDGF-D, CCL1 and CCL24 that may be of importance in CRC progression and may be of clinical value together with TNM stage in establishing prognosis.

SVENSK SAMMANFATTNING

Kolorektal cancer är en tumör i kolon eller rektum. I Sverige diagnosticerades år 2014 ca 6300 individer med denna cancertyp och ca 2550 personer dör årligen till följd av kolorektal cancer. Operation är det huvudsakliga behandlingsalternativet för kolorektal cancer och vid fjärrmetastaser är överlevnaden < 10 %. Det är därför viktigt att hitta markörer som tillsammans med TNM-stadium kan ge tidig information om sjukdomens prognos och lämplig uppföljning av patienter.

Utveckling av kolorektal cancer sker genom ackumulering av genetiska mutationer och epigenetisk nedreglering av tumörsuppressorgener. Därutöver spelar interaktionen mellan tumören och dess närmaste omgivning, innehållande tillväxt- och inflammatoriska faktorer, en viktig roll i tumörens utveckling och metastasering.

Syftet med avhandlingen var att studera associationen mellan CD93, PLA2G4C, PDGF-D samt inflammatoriska cytokiner och kolorektal cancer progression.

En prospektiv studie visade att *CD93* och *PLA2G4C* SNP var potentiellt viktiga förbedömning av kolorektal cancer prognos. T/T genotypen av SNP rs2749817 i *CD93* var associerad med högre uttryck av CD93 i kolorektal cancer vävnad, främst bland patienter i stadium IV. Därutöver observerades fler återfall efter operation hos patienter med T/T genotypen. A allelen hos *PLA2G4C* SNP rs1549637 är en möjligtvis bättre markör för cancerspecifik överlevnad vid stadium II än faktorer som idag används för att selektera patienter till adjuvant behandling. Sammantaget antyder detta att T/T genotypen av *CD93* och A allelen av *PLA2G4C* kan vara genetiska markörer relaterade till allvarlig tumörsjukdom och spridning. Därutöver kan de eventuellt selektera patienter som kräver tätare uppföljning och adjuvant behandling.

För att studera den förmodade inblandningen av PDGF-D i kolorektal cancer undersöktes dess effekt på PDGF-D signalering *in vitro*. PDGF-D signaleringen förändrade genexpressionen av gener involverade i tumörutveckling och spridning, vilken kunde blockeras av tyrosinkinashämmaren imatinib. Det antyder att PDGF-D signalering kan vara en viktig faktor vid kolorektal cancer progression och ett potentiellt mål för behandling.

Analysen av ett flertal inflammatoriska cytokiner visade en korrelation mellan höga cytokinnivåer och ökad cancerspecifik och total dödlighet två år efter operation. Höga CCL1 och CCL24 nivåer var de enda faktorerna som förblev signifikant associerade med cancerspecifik mortalitet vid fördjupad statistisk analys och bör studeras vidare.

Sammanfattningsvis presenterar denna avhandling cirkulerande och genetiska faktorer såsom CD93, PLA2G4C, PDGF-D, CCL1 and CCL24 som eventuellt är viktiga vid bedömning av kolorektal cancer progression tillsammans med TNM stadium.

LIST OF PUBLICATIONS

The thesis is based on the following publications and manuscripts, referred to in roman numerals in the text.

- I. **Olsen RS**, Lindh M, Vorkapic E, Andersson RE, Zar N, Löfgren S, Dimberg J, Matussek A, Wågsäter D. CD93 gene polymorphism is associated with disseminated colorectal cancer. *Int J Colorectal Dis.* 2015;30(7):883-90.
- II. **Olsen RS**, Andersson RE, Zar N, Löfgren S, Wågsäter D, Matussek A, Dimberg J. Prognostic significance of PLA2G4C gene polymorphism in patients with stage II colorectal cancer. *Acta Oncol.* 2016;55(4):474-9.
- III. **Olsen RS**, Dimberg J, Geffers R, Wågsäter D. Possible role and therapeutic target of PDGF-D signalling in colorectal cancer. Manuscript is under review in BMC Cancer.
- IV. **Olsen RS**, Nijm J, Andersson RE, Dimberg J, Wågsäter D. Circulating inflammatory factors associated with worse long-term prognosis in colorectal cancer. Manuscript is under review in World Journal of Gastroenterology.

ABBREVIATIONS

α	Alpha
AA	Amino acid
AJCC	American Joint Committee on Cancer
APC	Adenomatous polyposis coli tumour suppressor gene
ATP	Adenosine triphosphate
β	Beta
CAM	Cell adherence molecule
CCL	Chemokine C-C motif ligand
CCR	C-C motif chemokine receptor
CD4 ⁺	Cluster of differentiation 4-positive
CD93	Cluster of differentiation 93
cDNA	Complementary deoxyribonucleic acid
CEA	Carcinoembryonic antigen
CEACAM5	Carcinoembryonic antigen related cell adhesion molecule 5
CIS	Chromosome instability pathway
COX-2	Cyclooxygenase 2
CRC	Colorectal cancer
cRNA	Complementary RNA
CVD	Cardiovascular disease
CXCL	Chemokine C-X-C motif ligand
CX3CL1	C-X3-C motif chemokine ligand 1

DG	Dystroglycan
ECM	Extracellular matrix
EGF	Endothelial growth factor
EGFR	Endothelial growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial to mesenchymal transition
FAP	Familial adenomatous polyposis
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GI	Gastrointestinal tract
GTP	Guanosine triphosphate
HAS2	Hyaluronan synthase 2
HNPCC	Hereditary nonpolyposis colorectal cancer
HRP	Horseradish peroxidase
IHC	Immunohistochemistry
IL	Interleukin
IFN- γ	Interferon gamma
KRAS	Kirsten sarcoma viral oncogene homolog proto-oncogene
LD	Linkage disequilibrium
LOX	Lysyl oxidase
MMP	Matrix metalloproteinase
mRNA	messenger RNA

MSI	Microsatellite instability pathway
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
phPDGFR	Phospho-specific platelet-derived growth factor receptor
PE	Phycoerythrin
PGE2	Prostaglandin E2
PLA2	Phospholipase A2
PLA2G2A	Phospholipase A2 group II A
PLA2G4	Phospholipase A2 Group IV
PLA2G4A/C	Phospholipase A2 Group IV A/C
PPAR	Peroxisome proliferator-activated receptor
PVDF	Polyvinylidene fluoride
qPCR	Real-time polymerase chain reaction
REMARK	Recommendations for tumour marker prognostic studies
RPLP0	Ribosomal protein lateral stalk subunit P0
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Serpin E1	Serpin family E member 1
SNP	Single nucleotide polymorphism
Taq	<i>Thermus aquaticus</i>
TBP	TATA-box binding protein
Th	T helper
TNF	Tumour necrosis factor
TNFSF4	TNF super family member 4

TNM	Tumour, node, metastasis
TP53	Tumour suppressor P53
VCAN	Versican
VEGF	Vascular endothelial growth factor
WB	Western Blot

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INTRODUCTION

The large intestine: colon and rectum

Colon and rectum together with the anus are parts of the large intestine (approximately 1.5 m in length) and are the final section of the gastrointestinal tract (GI) (Figure 1). The large intestine consists of several segments, including caecum, ascending colon, transverse colon, descending colon, sigmoid colon, rectum and anus (Irving and Catchpole, 1992).

Figure 1: Overview of the large intestine modified from Irving and Catchpole (Irving and Catchpole, 1992). 1: caecum; 2: ascending colon; 3: transverse colon; 4: descending colon; 5: sigmoid colon; 6: rectum; 7: anus.

The colon is located in the peritoneal cavity while the rectum is located within the pelvis. The main function of the colon include to mix the colonic contents back and forth to ensure absorption of minerals, nutrients and water as well as storage of faeces (Greenwood-Van Meerveld et al., 2017). The rectum is ~12 cm in length (Cancer, 2012) and its main functions are temporary storage of faeces and defecation (Greenwood-Van Meerveld et al., 2017).

Like the rest of the GI the tissue of the large intestine is composed of four main layers. The innermost layer is the mucosa, which consists of an epithelial layer with intestinal crypts and lymphoid nodules and is surrounded by the muscularis mucosae.

The second layer is the submucosa, which contains nerves, blood vessels and connective tissue, followed by the third layer, the muscularis propria, which consists of two layers of smooth muscle cells. The fourth layer is the serosa consisting of connective tissue covered with squamous cell epithelium (Cancer, 2012).

Colorectal cancer

Colorectal cancer (CRC) is defined as a cancer appearing in the colon or in the rectum due to their adjacent anatomical locations, but the term is still debated. Several studies have shown genetic, morphological, and clinical differences, as well as differences in survival between cancers of the colon and rectum (Kornmann et al., 2013, van der Sijp et al., 2016) suggesting that CRC might consist of two separate entities. As an example Kornmann *et al.* showed an improved survival in treated patients with colon cancer but not among patients with rectum cancer. Also, a higher occurrence of lung metastases among patients with rectal cancer was found. The conclusion of the study was that there might be differences in chemosensitivity between the colon and rectum (Kornmann et al., 2013). Another study by van der Sijp *et al.* investigated differences in characteristics, prevalence of complications, survival as well as the rate of recurrence among patients with colon- and rectal cancer. This study showed a lower survival among colon cancer patients because of a more severe effect of complications compared with patients with rectal cancer (van der Sijp et al., 2016).

On a world basis about 1.4 million cases of CRC were reported in 2012 and around 700.000 individuals passed away due to CRC (Ferlay et al., 2015). In Sweden, CRC is the third most common form of malignancy (Norr, 2016) and in 2014 about 6300 individuals were diagnosed with an equal distribution among men and women (Cancerfonden, 2016). Approximately 2550 individuals diagnosed with CRC die each year due to their CRC (Norr, 2016).

Three categories of CRC can be distinguished by their forms of origin and expression. Sporadic CRC appears in individuals that carries mutations in the tissue but do not have any family history of CRC. This category is assumed to include 60-80 % of CRC cases (Watson and Collins, 2011). The second form is the family type of CRC and constitutes 20-40 % of CRC cases. This category is based upon population studies that have shown an increased chance of developing CRC when family members of primary consanguinity have suffered from the sporadic form of CRC. Nevertheless, it is thought that this category may be a result of both environmental as well as hereditary factors. The hereditary type is the third category of CRC, which can be distinguished by the presence of adenomatous polyps (Arvelo et al., 2015).

Carcinogenesis of CRC

Carcinogenesis, also named oncogenesis or tumourigenesis, is a complicated process by which cancers are generated and is a multistep mechanism occurring due to accumulation of errors in vital regulatory pathways. The process starts in a single cell which then multiplies and acquires additional changes that give the cell a survival advantage over its surrounding cells. This altered cell must then be amplified to generate a large number of cells that constitute a cancer (King and Robins, 2006b).

CRCs are slowly growing cancers that start developing as a tumour or as a tissue growth on the inner lining of the colon or rectum. If the growth is abnormal, i.e. a polyp that eventually becomes cancerous, it can form a tumour on the wall of the colon or rectum, and subsequently grow into blood vessels or lymph vessels, increasing the risk of developing metastasis to other sites of the human body (Vogelstein and Kinzler, 1993). In CRC there are two major pathways in carcinogenesis: the chromosome instability pathway (CIS) and the microsatellite instability pathway (MSI). The CIS is the pathway of sporadic CRC through mutations of the adenomatous polyposis coli suppressor gene (APC), the Kirsten sarcoma viral oncogene homolog proto-oncogene (KRAS) and the tumour suppressor P53 (TP53) gene. MSI is mainly the pathway for hereditary nonpolyposis colorectal cancer (HNPCC) through mutations in mismatch repair genes (Raskov et al., 2014).

The concept of carcinogenesis was presented in a model for CRC formation developed by Fearon and Vogelstein in 1990 and consists of the following phases: initiation, promotion and progression from pre-existing benign and slow-growing tumours (adenomas), into carcinomas and metastatic CRC through specific genetic events (Figure 2) (Fearon and Vogelstein, 1990). Still, there are alternative pathways including MSI but CIS is the most commonly used to explain the adenoma-carcinoma development in CRC (Raskov et al., 2014).

Figure 2: A simplified version of Fearon and Vogelstein’s genetic model for CRC carcinogenesis modified from Fearon and Vogelstein (Fearon and Vogelstein, 1990). Red arrows denote tumour suppressor genes that are mutated or down-regulated during development of CRC from normal epithelium with metastasis as the end of the model resulting in spread of cancer to the most common distant organs, the liver and lungs. Mutation of APC increases cyclooxygenase 2 (COX-2) activity.

It is assumed that approximately 85 % of all CRCs develop through CIS and the most important mutations are in *APC*, *KRAS* and *TP53* (Raskov et al., 2014) although other genes also are involved in the CIS pathway.

APC is an important tumour suppressor gene assumed to be a “gate keeper” gene since mutation of *APC* is thought to be the gate towards malignant transformation. The functions of *APC* are regulation of cell orientation, intracellular communication, proliferation and transcription. The main function is regulation of the Wnt-signalling pathway by its interaction with β -catenin (Fearon and Vogelstein, 1990, Raskov et al., 2014) and loss of or mutation of *APC* results in activation of the Wnt-signalling pathway leading to the initial step

from normal to hyper proliferative epithelium (Fearon and Vogelstein, 1990). The gene product, the APC protein, ensures the function of the important junctions between colonocytes ensured by cell adherence molecules (CAMs) the cadherins. To maintain the proper function of the cadherins the APC protein must bind to the cadherin molecule together with β -catenin and GSK3- β . This binding to the cadherin molecule ensures the normal function of the junctions. The binding of β -catenin to the cadherin complex then ensures low levels of free β -catenin in the cytoplasm and is important since β -catenin otherwise will translocate into the nucleus and upregulate the Wnt-signalling pathway and result in an accelerated proliferation, impaired differentiation and apoptosis leading to the formation of adenomas (Mann et al., 1999, Raskov et al., 2014). Mutation of *APC* also results in increased COX-2 activity that is maintained by its production of prostaglandin E2(PGE2) contributing in promoting cancer growth (Wang and DuBois, 2013, Raskov et al., 2014).

KRAS is an oncogene with its natural form as a proto-oncogene, and is thought to play an important role in the transition from adenoma to carcinoma (Raskov et al., 2014). *KRAS* is a short gene sequence susceptible to point mutations and substitution of single amino acids (AAs) in a nucleotide that can lead to activation of mutation. *KRAS* mutations are found in approximately 30-50 % of CRC cases and gives the colonocytes an advantage in growth as guanosine triphosphate (GTP) activity is lost with mutation. The increased levels of GTP result in constant signalling. The gene product of *KRAS*, KRAS protein, is responsible for the transduction the mitogenic signals from growth factor receptors on the cell surface to the cell nucleus (Dobre et al., 2013). In an active form, KRAS can affect several cellular pathways that control apoptosis, cellular growth, differentiation, organization of the cytoskeleton, cell motility, inflammation and proliferation (Pino and Chung, 2010). The *KRAS* mutations are often seen in early dysplasia and in hyperplastic polyps. If the mutations occur after an *APC* mutation the dysplastic lesions are often progress towards cancer (Raskov et al., 2014).

TP53 plays a crucial role in all cells since it controls cell cycle, apoptosis via its protein product P53, which induces cell cycle arrest and facilitates DNA repair due to replication errors or mutations, and if the DNA repair is unsuccessful P53 induces apoptosis. In most tumours the mutated form of *TP53* occurs due to missense mutation, which inactivates the transcriptional activity of P53. *TP53* is usually found to be commonly mutated in CRC and is

thought to be important in the transition of large adenomas into invasive carcinomas (Raskov et al., 2014).

Even though CIS is considered the major pathway in the development of sporadic CRC, this does not explain why the majority of adenomas never progress to invasive carcinomas. This might be due to other, unknown pathways enabling the cells to overcome CIS (Raskov et al., 2014).

Development of metastasis

Metastasis is the spread of cancer cells from the primary tumours into the surrounding tissues and distant organs. The liver and lungs are the most common distant organs for CRC metastases (Chambers et al., 2002). Of all CRC patients, approximately 25 % have a metastatic disease at the time of diagnosis, and a high number of patients will carry undetectable micro-metastases. Moreover, another 25 % of all CRC patients are at the risk of developing metastatic tumours during the course of the disease (Kindler and Shulman, 2001).

The development of tumour metastasis is a complex process involving a series of events leading to the spread and growth of cancer cells to secondary sites of the human body (Figure 3).

Figure 3: Events during the development of tumour metastases modified from King and Robins and Alizadeh et al. (King and Robins, 2006a, Alizadeh et al., 2014). 1: Primary tumour developed by either diverse genetic factors or gene alterations; 2: Angiogenesis, ensuring a transition from a pre-vascular lesion to vascularized and progressively outgrowing cancer cells; 3: Dissociation; 4: Invasion; 5: Intravasation; 6: Extravasation; 7: Spread and growth of cancer cells to secondary sites such as the liver and lungs.

The disassociation phase (Figure 3) is thought to be the initiating step in which cancer cells are detached from the primary tumour due to changes in the cell-extracellular matrix (ECM) and cell-cell adherence, followed by their migration into the surrounding tissue through the basement membrane and the ECM (King and Robins, 2006a). The cell-cell signalling and the adherence in cancer cells are mainly accomplished by CAMs, such as cadherins, integrins and selectins, which contributes to both cellular adhesion and motility. These are deregulated in epithelial cancers and results in single migrating cancer cells (Alizadeh et al., 2014).

These cellular changes are defined as epithelial to mesenchymal transition (EMT). This is an important step of the invasion phase of metastasis but is also important in angiogenesis. EMT can be induced by different signals from tyrosine kinases such as endothelial growth factor (EGF), platelet-derived growth factor D (PDGF-D) and fibroblast growth factor (FGF)

leading to a remodeling of the actin cytoskeleton and scattering of cancer cells (Thiery, 2002).

The cancer cells then form a finger-like protrusion called a pseudopod at their leading edge that establishes a binding to the ECM via integrins. The attachment of integrin to the ECM ensures an invasion and migration of the cancer cells through the ECM towards the lymphatic- and vascular circulation (Figure 3). The integrins enables also attachment to proteases such as serine proteases, matrix metalloproteinases (MMPs). The MMPs are then activated, resulting in a proteolysis of specific parts of the ECM, creating a path for the cancer cells through the ECM. The MMPs may also be activated by cytokines. The cancer cells attach to the vascular endothelial cell wall and by changing their shape they are able to penetrate into the endothelial cell-cell junctions. Additionally, the modulation of endothelial cell barrier permeability by macrophage-derived tumour necrosis factor alpha (TNF- α) may also contribute in this process called intravasation (Figure 3). Cancer cell overexpression of vascular endothelial growth factor (VEGF) may also contribute by inducing endothelial barrier disruption (King and Robins, 2006a, Alizadeh et al., 2014).

Inside the bloodstream, single cancer cells are likely to be destroyed by actions of the surveillance by the host immune system. Cancer cells may avoid this destruction by travelling together in clusters, interacting with platelets, macrophages, leukocytes and the vascular endothelium by binding of CAMs (e.g. selectin and integrin) forming an emboli. The passive displacement of cancer cells is also influenced by cytokines. The emboli can then attach to the endothelial cell lining of the blood vessel wall by binding of the cancer cells' CAMs to the glycoproteins (laminin, collagen, fibronectin) expressed on the endothelial cell surfaces. Once attached the cancer cells can progress their metastatic process by destroying the basement membrane of the circulation system with the help of proteases, a process called extravasation (Figure 3) (King and Robins, 2006a, Alizadeh et al., 2014).

After extravasation the cancer cells migrate to their growth site, which requires decomposition of the ECM, and that the cells reach bigger blood vessels ensuring movement towards distant organs such as the liver and lungs (Figure 3). Once the cancer cells have

reached distant organs and are attached they need to form new blood vessels in a process called angiogenesis to gain a supply of essential nutrients as well as oxygen to enable further growth and establishment of metastasis (King and Robins, 2006a).

Angiogenesis

Angiogenesis is a rate-limiting step determining whether or not a metastasis remains in a dormant state or if it grows further on. Even though angiogenesis is associated with the establishment of secondary tumours it is also an essential process in the establishment of primary tumours. The term angiogenesis describes the growth of new capillary blood vessels by sprouting, in which intestinal tissue columns are inserted into the lumen of pre-existing vessels and partition the vessel lumen (King and Robins, 2006a) and plays a crucial role in supplying oxygen and other important nutrients necessary for the growth and progression of tumours (Kumar et al., 2016).

The process of angiogenesis consists mainly of three phases. It is initiated by increased tumour secretion of tumour-derived growth factors, such as PDGFs, FGFs, transforming growth factors, EGFs and VEGFs, which stimulate local endothelial proliferation inside the capillaries enabling them to sprout and penetrate through the ECM and move in the direction of the tumour (Figure 4) (Kumar et al., 2016, King and Robins, 2006a).

Figure 4: The initial and proliferative phase of angiogenesis modified from King and Robins and Kumar et al. (King and Robins, 2006a, Kumar et al., 2016). 1: tumour (blue cell cluster) secretes growth factors (coloured spots) and initiates angiogenesis; 2: growth factor stimulated endothelial cells proliferate inside the capillaries enabling sprouting and penetration through the ECM facilitating movement towards the tumour.

This invasive and proliferative phase requires remodelling of the ECM through protease digestion of the existing glycoproteins followed by formation of a new ECM and altered ECM contacts as well as directional chemotactic stimuli to promote the endothelial sprout towards the cancer. These events occur in several capillary sites leading to a fusion of the separate sprouts to form a capillary network in and around the cancer. In this final phase the endothelial cells mature and differentiate, a basement membrane is formed and other cells of importance such as smooth muscle cells are recruited, which contribute to the maintenance of the new vessels (King and Robins, 2006a, Pietrzyk, 2016). Since angiogenesis is an important process in cancer development it is also an important target for anti-angiogenic therapy to enable a decrease in cancer cell growth and metastasis (Kumar et al., 2016).

Symptoms and risk factors

The symptoms associated with suspected CRC development are blood in the faeces, hard or loose faeces, no appetite, weight loss, tiredness and a slow development of anaemia (Norr, 2016, Register, 2013). Even though most cases of CRC arise sporadically (Amersi et al., 2005) there are several risk factors associated with CRC development and they can be divided into two groups: controlled risk factors, including environment and life style, and those that cannot be controlled, such as hereditary factors, previous colonic polyps and age (Hagggar and Boushey, 2009).

Hereditiy

Hereditiy of tumour development is thought to be one potential risk factor for contracting CRC even though most CRCs are assumed to be sporadic. In about 4 % of all CRC cases the cancer is accounted for by HNPCC, also named Lynch syndrome. It is the most common hereditary form and patients with HNPCC have an increased risk of developing other cancers (Hampel et al., 2008). Even though HNPCC-affected individuals can develop colonic adenomas with a greater frequency than the normal population, polyps rarely develop (Jasperson et al., 2010). Familial adenomatous polyposis (FAP) is the second most inherited CRC syndrome and it is characterized by the development of multiple colonic adenomas. It is a less severe form of CRC disease and the development of FAP starts in early adolescence (Jasperson et al., 2010, Arvelo et al., 2015). In the absence of preventive surgery FAP may develop into a malignant form (Arvelo et al., 2015) and the average age at diagnosis if untreated is 39 years (Jasperson et al., 2010).

Lifestyle and age

The diet is considered as a major risk factor for CRC. High consumption of red meat, food containing high levels of animal fat and a low level of dietary fiber, as well as processed food have been associated with increased risk of developing CRC (Hagggar and Boushey, 2009, Watson and Collins, 2011). Other lifestyle-related risk factors for CRC are smoking, high alcohol consumption, obesity and physical inactivity (Hagggar and Boushey, 2009).

Age and the development of CRC are considered to go “hand in hand”, indicating that risk of developing CRC increases with age due to accumulations of DNA damage over long time periods (Register, 2013). In Sweden, about 80 % of the patient group are 65 years or older when they get the CRC diagnosis (Cancerfonden, 2016).

Concomitant diseases

Since CRC mostly affects older patients, the risk also increases for development of other underlying diseases and about 40 % of CRC patients have concomitant diseases such as cardiovascular disease (CVD), diabetes and other malignancies (Edwards et al., 2014).

CVD and CRC are both diseases that are increasing in incidence and prevalence, in part due to the aging population. Lifestyle, such as physical inactivity and an unhealthy diet are common risk factors for both CVD and CRC (Yang et al., 2010) and affect mainly elderly patients within similar age groups (65 years or older) (Robertson et al., 2010). Also, a higher risk of abnormal intima/media thickness of carotid arteries among patients with colorectal adenomas and adenocarcinomas has been seen (Cha et al., 2011).

Inflammatory bowel disease is a chronic inflammatory disorder of the GI divided into Crohn`s colitis and ulcerative colitis (Qu et al., 2017). Crohn`s colitis has been associated with increased risk of developing cancer of the colon (von Roon et al., 2007), while the risk of developing CRC has been shown to increase with duration of ulcerative colitis, 2 % at 10 years and 18 % by 30 years (Eaden et al., 2001). Summarized, the appearance of the colitis associated inflammation seem to accelerate the progression from chronic colitis to CRC development (Qu et al., 2017).

Prognosis

The medical term 'prognosis' is used to indicate the probable direction, outcome, as well as an estimation of the aggressiveness of a disease. In cancer, prognosis is measured by the probability of staying alive or without recurrence of the disease for a certain amount of time after diagnosis, or at the start of adjuvant treatment. Often a threshold of five years is used for the survival or the cancer re-growth, but depending on the treatment and the disease, shorter or longer time periods might be considered (Parkin and Hakulinen, 1991).

Pathological stage is the most important estimation of CRC prognosis. The American Joint Committee on Cancer (AJCC) introduced the tumour-, node- and metastasis (TNM) staging system, which is more differentiated than the previous Duke's stage system (Table 1) (Cancer, 2012). The TNM system is based on local tumour depths of invasion (T1-4), the presence of and the amount of lymph node metastases (N0-2), as well as the presence of distant metastases (M0-1) (Hutter, 1987). Today the seventh version of the TNM staging system published in 2010 is used by clinicians (Table 1) (Cancer, 2012).

The prognosis of CRC is related to the TNM stage at diagnosis with a five-year survival rate of 90 % at early diagnosis resulting in better prognosis and less than 10 % if distant metastases have developed (Coppede et al., 2014). Among patients with stage II-III CRC that have undergone intended curative surgery, 20-60 % would be at risk of cancer recurrence (Mosolits et al., 2005, Tebbutt et al., 2002). Also, if the disease disseminates extensively and the chance of additional curative surgery is eliminated, only palliative treatment remains for the patients.

Table 1: Classification of CRC based upon Duke`s- and TNM stage classification systems modified from the AJCC Cancer Staging Atlas (Cancer, 2012) and survival rate modified from the American Cancer Society (The American Cancer Society, 2017).

Duke`s stage system	Stage grouping	TNM stage system	Comments	5 year prognosis •
	0	Tis, N0, M0	Tis: Carcinoma <i>in situ</i> ; intraepithelial or invasion of lamina propria N0: No regional lymph node metastasis M0: No distant metastasis	
A	I	T1, N0, M0 T2, N0, M0	T1: Tumour invades submucosa T2: Tumour invades muscularis propria	~ 87-92 %
B	II A	T3, N0, M0	T3: Tumour invades through the muscularis propria into pericorectal tissues	~ 80-87%
	II B	T4a, N0, M0	T4a: Tumour penetrates through the surface of the visceral peritoneum	~ 49-63 %
	II C	T4b, N0, M0	T4b: Tumour directly invades or is adherent to other organs or structures	
C	III A	T1-T2, N1/N1c, M0 or T1, N2a, M0	N1: Metastasis in 1-3 regional lymph nodes N1c: Tumour deposite(s) in the subserosa, mesentry, or non-peritonealized pericolic or perirectal tissues without regional nodal metastasis	~ 84-89 %
	III B	T3-T4a, N1/N1c, M0 or T2-T3, N2a, M0 or T1-T2, N2b, M0	N2a: Metastasis in 4-6 regional lymph nodes N2b: Metastasis in ≥7 regional lymph nodes	~ 69-71 %
	III C	T4a, N2a, M0 or T3-T4a, N2b, M0 or T4b, N1-N2, M0		~ 53-58 %
D	IV A	Any T, any N, M1a	M1a: Metastasis confined to one site or organ	~ 11-12 %
	IV B	Any T, any N, M1b	M1b: Metastasis in more than one site/organ, or the peritoneum	

▪ Based upon survival rates (%) for colon and rectum separately.

Diagnosis and treatment

Diagnosis of CRC is based upon the results from colonoscopy or sigmoidoscopy and tumour biopsies from individuals with CRC-associated symptoms (Cunningham et al., 2010). Also, levels of carcinoembryonic antigen (CEA) may be a preoperative support in preoperative staging and planning of surgery (Labianca et al., 2010).

The pretreatment phase for newly diagnosed CRC includes physical examination and a complete colonoscopy to detect any possible metachronous tumours. Computed tomography of the chest, abdomen and pelvis to localize the tumour and to identify any metastatic disease is also part of the pretreatment strategy (Norr, 2016). In patients with rectal cancer, the assessment of local tumour extension is important for optimal treatment. High resolution magnetic resonance imaging is used to measure the spread in the surrounding epithelium more accurately and to assess the resection margin between the edge of the tumour and the surrounding tissue in the rectum (Norr, 2016). Based on the result of these examinations preoperative treatment with chemo- and/or radiotherapy can be initiated before surgical removal of the cancer (Norr, 2016).

Surgery is the main treatment option in the treatment phase. During surgery of cancer located in the colon or rectum a total resection (R0 resection) of the tumour should be performed with adequate margins and removal of regional lymph nodes. This is defined as radical surgery. Also, margins of ≥ 5 cm around the cancer are recommended. At least 12 lymph nodes should be removed and these nodes should be analyzed to allow appropriate nodal staging since analysis of fewer than 10 lymph nodes may under-stage the tumour (Cunningham et al., 2010, Norr, 2016). The removal of lymph nodes is essential since spread of tumour cells through lymph nodes is of importance in development of metastasis. Also, the removal of lymph nodes may ensure a lower risk of recurrence (Norr, 2016).

After surgery and examination of the specimen by a pathologist the TNM stage of the resected tumour can be decided to determine the treatment strategy and follow-up of the patient. Decisions on treatment and follow-up of CRC patients are made by a multidisciplinary group of clinicians. The choice of optimal treatment for the CRC patients depends on tumour location, differentiation grade of the tumour, TNM stage, histopathological type, presence of infiltrating blood vessels and lymph nodes, age, condition of the patient, as well as risk of recurrence. The postoperative treatment option apart from surgery is adjuvant treatment, which may consist of chemotherapy, radiotherapy, targeted therapy and liver resection (Labianca et al., 2010, Norr, 2016). The goal with adjuvant treatment is to decrease tumour growth and ultimately its eradication.

Imatinib

Imatinib (Glivec®, Gleevec®) is a drug that has been approved for treatment of chronic myeloid leukaemia and gastrointestinal stromal tumours (Druker et al., 2001, Demetri et al., 2002) and was the first class of agents that acted by inhibition of specific tyrosine kinases, rather than killing all rapidly dividing cells (Ohno, 2006). Treatment with imatinib show only mild to moderate levels of side effects such as diarrhoea, rash, fatigue and nausea (Demetri et al., 2002).

The drug has been shown to inhibit the activity of several tyrosine kinases that play important roles in cell growth, motility and survival (Popow-Wozniak et al., 2011) such as breakpoint cluster region-Abelson1 (Schindler et al., 2000), c-Kit, and platelet-derived growth factor receptors (PDGFRs) such as PDGFR-beta (PDGFR- β) (Moawad, 2015). In short, imatinib functions by binding to and inducing a blockage of the adenosine triphosphate (ATP)-binding pocket of the tyrosine kinases preventing access of ATP, which is of importance for tyrosine activity. Consequently, imatinib shuts down all downstream signalling from the tyrosine kinases affecting the cell survival (Popow-Wozniak et al., 2011). In CRC, cell studies have shown that imatinib treatment induces apoptosis and inhibition of

cancer cell migration, suggesting that imatinib could be a potential drug in CRC therapy (Popow-Wozniak et al., 2011).

Markers in blood and tissue

According to the National Institute of Health, the term ‘biomarker’ refers to a biological molecule found in blood, in other body fluids, or in tissues that is a sign of normal or abnormal processes, or of a condition or a disease, or even of responses of therapeutic intervention (De Gruttola et al., 2001).

In cancer such as CRC, it is important to identify biomarkers that allow detection of the disease at an early stage, which may lead to an early diagnosis, establishment of a personalized treatment strategy and improvement of prognosis. Additionally, identification of biomarkers in serum and blood is preferable since sequential blood tests are non-invasive and more accessible than repeated tissue biopsies in a clinical setting. There are several suggested biomarkers for CRC available such as the fecal marker faecal occult test and genetic markers such as *KRAS* and serum markers such as CEA (Locker et al., 2006, Labianca et al., 2010, Norr, 2016).

Many studies are focusing on finding new potential biomarkers useful in establishing cancer prognosis. The reporting recommendations for tumour marker prognostic studies (REMARK) is a checklist consisting of twenty items that are important to report on when publishing tumour marker prognostic studies. These recommendations should ensure a more consistent, high quality reporting of tumour marker studies and applies in general to prognostic factors e.g. biological molecules, size of tumour, presence of tumour cells in regional lymph nodes, abnormal features of cells, gender and age. It also applies to other diseases than cancer. REMARK can be used in any studies involving prognostic factors regardless if those prognostic factors are biological markers, clinical assessments, imaging assessments or measures of functional status in activities of daily living (Altman et al., 2012).

Carcinoembryonic antigen

CEA is a high molecular weight glycoprotein that belongs to the immunoglobulin superfamily and is the oldest and most widely used serum marker in CRC. CEA is a recommended soluble biological marker used in surveillance following curative surgery of the primary CRC tumour, and measurement of CEA is quite inexpensive and has less disadvantages for the patients (Duffy et al., 2007).

In the clinic, CEA may be useful in both preoperative staging and planning of surgery. Also, postoperative CEA levels are recommended to be checked every three months for stage II and III CRC, for at least three years if the patient is a potential candidate for surgery or chemotherapy for metastatic CRC. On the other hand, the problem with CEA is its low predictive value for diagnosis in asymptomatic patients since it has a relatively low sensitivity and specificity. This explains why elevated CEA levels (>5 mg/mL), which may correlate with poorer prognosis among CRC patients, is thought to be insufficient for determining whether or not patients should receive adjuvant treatment (Labianca et al., 2010, Locker et al., 2006).

Kirsten sarcoma viral oncogene homolog proto-oncogene

In CRC metastasis mutation of RAS genes are frequent and mutations of *KRAS* are most common (~ 40 %). The RAS proteins effectuates intracellular signals downstream the endothelial growth factor receptor (EGFR) and specific mutations of the RAS genes results in modified proteins which induce a constant activation of pathways regulating tumour cell growth and proliferation through EGFR. This activation cannot be efficiently blocked by chemotherapy and antibody targeted treatment (Karapetis et al., 2008). In CRC with RAS mutations a combination of chemotherapy and antibody targeted treatment may give a prolonged survival of only 1-2 months. However if the RAS mutation is wild-type antibody targeted treatment or in combination with chemotherapy has shown to be more efficient in inhibiting EGFR signaling resulting in a prolonged survival of 30 months (Norr, 2016).

Single nucleotide polymorphisms

Single nucleotide polymorphisms (SNPs), also named gene polymorphisms or genetic variants, are single base-pair positions in the genomic DNA that differ from one individual to another and can easily be analyzed in whole blood samples from individuals. To be defined as a SNP the least frequent allele should have a frequency of >1 % in the population (Kruglyak and Nickerson, 2001). Since autosomal regions carry one allele from the maternal chromosome and one from the paternal chromosome, an individual can exhibit one of three genotypes: homozygous for the major allele, or heterozygous- or homozygous for the minor allele. The definitions of the major- and the minor allele refers to the observed frequencies in a specific population (Crawford and Nickerson, 2005). There are two types of SNPs influencing the incidence of diseases. If the variation of an SNP is located within or close to the translated region, any AA substitutions which alter the protein synthesis may be directly connected to the disease. On the other hand, if a disease-associated SNP is located in a non-coding region or if there is no other gene near the SNP it is more difficult to determine the mechanism by which the SNP is associated with (Mimori et al., 2012).

Genetic variations, such as SNPs, are thought to play a role in individual variation in CRC susceptibility. In cancer, molecular characteristics might predispose tumours to a worse prognosis and their identification can enable the identification of patients at a higher risk of recurrence of the disease. Also, suitable predictive markers may also make it easier to choose the most appropriate therapy (Horvat et al., 2016). In CRC, several studies have focused on the effects of genetic variants and their association with CRC disease. As an example Chang *et al.* found that a polymorphism of stromal derived factor-1 α , which can promote cancer cell migration, was associated with lymph node metastasis in CRC and suggested that stromal derived factor-1 α might be a predictive marker for lymph node metastasis in CRC patients (Chang et al., 2009). Even though promising results have been discovered among SNPs most of them need further validation in larger prospective clinical trials before they can be defined as biomarkers (Horvat et al., 2016).

Circulating inflammatory factors and their contribution in CRC

Primary CRC develops progressively over time through the accumulation of genetic mutations and epigenetic silencing of tumour suppressor genes. Also, evidence suggests that the development of CRC and CRC metastasis is also promoted by the interaction between tumour and stroma, the tumour microenvironment (Itatani et al., 2016). The tumour microenvironment contains several host cells that may suppress or promote aggressiveness of the cancer. Lymphocytes including macrophages, natural killer cells and regulatory T-cells are found in the surrounding stroma and will normally eradicate cancer cells but may also contribute to cancer progression (Mager et al., 2016, Itatani et al., 2016).

The majority of infiltrating lymphocytes are cluster of differentiation 4-positive ($CD4^+$) T helper (Th) cells and the Th cells are central in development of an immune response by activating antigen-specific effector cells and in recruitment of cells of the innate immune system such as macrophages. The $CD4^+$ T helper cells can be divided into Th1 and Th2 cells. The Th1 cells are primary responsible for activation and regulation of the development and persistence of cytotoxic T-cell response. The Th2 cells favour a predominantly humoral response and are responsible for reducing the activation of the cytotoxic T-cell response (Knutson and Disis, 2005, Murphy et al., 2008). During Th differentiation the cytokine environment is important. Th1 commitment relies on production of $TNF-\alpha$ and interferon gamma ($IFN-\gamma$) while Th2 commitment relies on production of interleukin (IL) 4 (Knutson and Disis, 2005).

The cytokine network consists of cytokines, chemokines, interferons and interleukins (Murphy et al., 2008). They are secreted membrane-bound proteins produced from cells of both the innate- and the adaptive immunity, enabling immune cells to communicate with each other (Alizadeh et al., 2014, Murphy et al., 2008). Cytokines are structurally and functionally similar to growth factors which fulfil their functions by binding to their specific cell membrane receptors present on non-cancerous cell and immune cells such as Th1 and Th2 cells that induce an inflammatory stimulus. The inflammatory stimuli enable the infiltration of inflammatory cells that either eradicate cancer cells or participate in functions facilitating cancer development by affecting the proliferative and invasiveness of the cancers

by inducing chemoattraction, inflammation and/or angiogenesis (Itatani et al., 2016, Mager et al., 2016). The cytokines can be divided into pro-tumourigenic (cytokines that facilitate cancer progression) and anti-tumourigenic (cytokines that inhibits cancer progression) cytokines. There are also cytokines that are both pro- and anti-tumourigenic (Figure 5) (Mager et al., 2016).

Figure 5: Examples of pro- (red circle) and anti-tumourigenic cytokines (blue circle) involved in CRC carcinogenesis described in Itatani et al. and Mager et al. Cytokines that are both pro- and anti-tumourigenic are placed in between the circles (Itatani et al., 2016, Mager et al., 2016).

This suggests that it is important to study the specific contribution of cytokines in CRC development and progression as well as their impact on survival among CRC patients. This may enable further knowledge in CRC carcinogenesis.

Cluster of differentiation 93

Human cluster of differentiation (CD93) is an EGF-like domain containing a transmembrane protein (Figure 6) (Nepomuceno et al., 1997), which appears in two forms; a cell-associated full-length form and a truncated soluble form (Greenlee-Wacker et al., 2011). CD93 is a member of the C-type lectin family, which also includes, among other selectins, natural killer cell receptors, dendritic cell receptors and thrombomodulin (Mayer et al., 2017, Petrenko et al., 1999). The production and release of the soluble form of CD93 seem to be mediated by MMPs, but even pro-tumourigenic cytokines such as TNF- α might be able to induce shedding of CD93 (Bohlson et al., 2005).

Figure 6: The structure of CD93 modified from Nepomuceno et al. and Kao et al. (Nepomuceno et al., 1997, Kao et al., 2012). N: N-terminus; S: signal peptide; CTLD: C-type lectin-like domain; E1-E5: EGF-like domains; M: mucin-like domain; TM: transmembrane domain; C: C-terminus.

The expression of CD93 is observed on a variety of cell types involved in the inflammatory cascade and haematopoiesis, but it is predominantly expressed in the vascular endothelium (Fonseca et al., 2001). CD93 seems to have a functional role in adhesion, migration and angiogenesis (Langenkamp et al., 2015, Orlandini et al., 2014, Kao et al., 2012).

According to observations in studies using cell- and animal models as well as in measurement in human specimens, CD93 has been shown to be involved in several diseases such as CVDs (van der Net et al., 2008, Malarstig et al., 2011). It has also been associated with different types of cancer such as breast cancer (Lee et al., 2009), glioblastoma (Langenkamp et al., 2015), CRC (Olsen et al., 2015) and nasopharyngeal carcinoma (Bao et al., 2016). In relation to cancer, CD93 has been associated with the risk of developing cancer (Lee et al., 2009). In our study, a CD93 SNP associated with poor prognosis and the risk of

cancer-specific death (Olsen et al., 2015), which is in agreement with other cancer studies (Langenkamp et al., 2015, Bao et al., 2016).

To be able to understand the biological mechanisms behind the potential role of CD93 in the different types of cancers, more functional studies are needed. Several studies on cancer have implied that CD93 induces angiogenesis due to its increased expression in vascular endothelial cells and adhesion of endothelial cells (Orlandini et al., 2014, Olsen et al., 2015, Bao et al., 2016). Also, CD93 seem to be important for proper endothelial cell migration and vascular function (Langenkamp et al., 2015).

The EGF-like domain of CD93 has been shown to be important in angiogenesis by binding to EGF receptors, which induces cellular signalling, facilitating the development of angiogenesis (Kao et al., 2012). Another recent study has identified another signalling pathway of importance for the function of CD93 in angiogenesis, which was activated due to the cooperation between CD93 and the important ECM adhesion molecule dystroglycan (DG) (Galvagni et al., 2016). DG is able to bind to several ECM ligands (Barresi and Campbell, 2006), is expressed on endothelial cells, and is involved in cell adhesion to the ECM (Hosokawa et al., 2002). In the study by Galvagni *et al.*, cooperative interactions between CD93 and DG were demonstrated, which promoted migration of endothelial cells and organization of endothelial cells into capillary structures which is important in the development of angiogenesis (Galvagni et al., 2016). Since CD93 seems to be an important regulator of angiogenesis, which is a key process in cancer development and spread, further studies on its function and regulation in CRC are of importance.

Phospholipase A2 group IV C

The phospholipase A2s (PLA2s) is a large group of enzymes that are divided into several families such as secretory, cytosolic and calcium-independent PLA2s. The enzymes of the PLA2 family are defined by their ability to catalyze fatty acid from the *sn*2 position of glycerophospholipids resulting in free fatty acids, such as arachidonic acid (Yarla et al., 2016). Arachidonic acid is a substrate for production of inflammatory mediators such as PGE2 (Wang and DuBois, 2013). PGE2 is also produced by COX-2 (Wang and DuBois, 2013, Yarla et al., 2016), which in high levels has been shown to affect important processes in cancer such as angiogenesis, apoptosis, cell migration, cell proliferation and tumour invasion (Wang and DuBois, 2013, Sheng et al., 1998).

Intracellular PLA2s are grouped as cytosolic PLA2s, also called the PLA2 group IV members (PLA2G4s), and they require increased calcium and phosphatidylinositol phosphate levels to locate them to the membrane to ensure their activity and the release of arachidonic acid. PLA2G4 may be regulated by various stimuli from among others cytokines, hormones, eicosanoids and growth factors. They have shown to be involved in inflammation-related disorders such as in asthma, sepsis and atherosclerosis and in cancer (Yarla et al., 2016).

In CRC, some PLA2G4s have been suggested to be of relevance in CRC development and progression. PLA2 group IV A (PLA2G4A) has been shown to be overexpressed in CRC tissue (Dimberg et al., 1998, Osterstrom et al., 2002). Also, a secretory PLA2, PLA2 group II A (PLA2G2A), has been suggested to be a prognostic determinant for selecting patients at high risk for cancer recurrence in stage II CRC who might benefit from adjuvant treatment (Buhmeida et al., 2009).

Another PLA2G4 member, phospholipase A2 group IV C (PLA2G4C), predominantly expressed in the human brain, heart and skeletal muscles (Asai et al., 2003) has been shown to contribute both in the cellular arachidonic acid metabolism and in phospholipid remodelling (Cummings, 2007, Murakami et al., 2003). It may therefore be linked to the COX-2 pathway to produce prostaglandins (Murakami et al., 2003). The exact activity of PLA2G4C expression in tumourigenesis is still unknown but it seems to play a role in breast

cancer cell chemotaxis and invasion (Tian et al., 2011). Polymorphic variants of genes may play a role as factors that mediate inflammatory response and favour CRC progression (Theodoropoulos et al., 2006). An SNP, rs1549637, of *PLA2G4C* has been associated with worse prognosis, especially in stage II CRC, and might be a potential prognostic biomarker in the planning of individual adjuvant treatment (Olsen et al., 2016). Since *PLA2G4s* have been shown to be associated with worse prognosis in stage II CRC, both *PLA2G2A* and *PLA2G4C* should be further investigated in larger CRC patient groups to evaluate their prognostic value in the clinic.

Platelet-derived growth factor D

The PDGFs act as possible mitogens for cells of mesenchymal origin such as fibroblasts and monocytes and are some of the many growth factors involved in both cell growth and division. The PDGF family consist of four monomeric variants, which in active form dimerize into five different isoforms: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD. The PDGFs function by binding to their receptors, PDGFR- α and PDGFR- β , which in active form exist in three isoforms composed of the homodimers PDGFR- $\alpha\alpha$, PDGFR- $\beta\beta$, or as a heterodimer PDGFR- $\alpha\beta$ (Fredriksson et al., 2004). Upon binding of PDGFs to their respective receptors, the PDGFRs dimerize which leads to auto-phosphorylation and an increased tyrosine kinase activity as well as binding affinity for signalling molecules. The signal is transduced into the cell to promote differentiation, migration and survival (Heldin et al., 1998, Heldin and Westermark, 1999).

PDGF-D is the latest and the least studied member of the PDGFs, which functions by binding either to the PDGFR- $\alpha\beta$ or the PDGFR- $\beta\beta$ (Figure 7) (Fredriksson et al., 2004) and seems to play a role in cancers such as breast cancer and CRC (Ahmad et al., 2011, Chen et al., 2016). In CRC, PDGF-D has been shown to exceed its cellular effects through PDGFR- β , which is mainly expressed in stromal cells and pericytes (Lindmark et al., 1993, Sundberg et al., 1993). PDGF-D is involved in regulation of cellular processes such as angiogenesis, apoptosis, transformation, invasion, metastasis, migration and proliferation (Ustach and Kim, 2005, Li

et al., 2003). Still, the roles and the precise mechanisms of PDGF-D in CRC have not been fully investigated.

Figure 7: PDGF-DD may bind to and activate its receptors, PDGFR- $\alpha\beta$ and PDGFR- $\beta\beta$, indicated by solid arrows. The figure is modified from Fredriksson et al. (Fredriksson et al., 2004).

In a recent study by Chen *et al.* the expression of PDGF-D was found in CRC tissue and the expression correlated with stage. By investigating the effects of PDGF-D *in vitro* they saw a positive regulation of Notch1, indicating that PDGF-D expression contributes to cancer growth, aggressiveness and angiogenesis, which seem to be partly mediated by activating the Notch1 signalling pathway. They also found that the expression of the Twist1 transcription factor, which plays a vital role in EMT transformation and enhancement of tumour metastasis, correlated with the expression of PDGF-D. They proposed that there might be a cross-talk between PDGF-D, Notch1 and Twist1 during the acquisition of EMT transformation in CRC cell models (Chen et al., 2016).

Since PDGF-D displays an oncogenic activity through binding to and activation of its receptor, PDGFR- β , this has led to the theory that inactivation of the PDGF-D/PDGFR- β signalling might have an important impact on CRC therapy. Tyrosine kinase inhibitors have been developed such as imatinib, which has been shown to inhibit the function of PDGFR- β (Buchdunger et al., 2000) and may also be a candidate for inhibiting the effects of PDGF-D in CRC.

AIMS OF THESIS

The overall aim of this thesis was to study the association of circulating and genetic factors with CRC disease progression.

The specific aims of this thesis were to:

- Investigate the expression of CD93 and the association of genetic variations within the CD93 gene with survival (Paper I).
- Investigate the expression of PLA2G4C and the association with a genetic variation within the PLA2G4C gene with survival (Paper II).
- Investigate the expression and localization of PDGF-D in human CRC tissue and to study the putative involvement of PDGF-D signalling in colorectal carcinogenesis (Paper III).
- Study the association of circulating factors, cytokines, at the time of CRC surgery with survival (Paper IV).

MATERIAL AND METHODOLOGICAL CONSIDERATIONS

MATERIAL

Study population

The CRC project at the Department of Laboratory Medicine, Region Jönköping County, Sweden is an ongoing project established in 1996. The project is a collaboration between the Department of Laboratory Medicine, the Department of Surgery and the Department of Clinical Physiology, Region Jönköping County. The external collaborators involved in the study are the Department of Natural Science and Biomedicine, the School of Health and Welfare, Jönköping University and the Division of Drug Research, the Department of Medicine and Health Sciences, Linköping University. The overall aim of the CRC project is to find risk factors revealing prognosis and survival among CRC patients at an early stage of the disease. This is done prospectively by comparing the laboratory results obtained in the study with the clinical covariates found in the patients' medical records.

Today the project consists of samples from >520 CRC patients who have undergone surgical resections for primary colorectal adenocarcinomas and >500 healthy blood donors (venous blood samples only) collected from blood bank centers in Region Jönköping County. Blood samples from blood donors serve as controls in the study. The CRC samples (matched normal and tumour tissue biopsies and blood samples) are continuously collected from the Department of Surgery, Region Jönköping County.

For each patient included in the project, tumour and adjacent normal tissue biopsies (~5 cm from the tumour) are excised and collected during surgery, and frozen at -70 °C until analysis. Also two venous blood samples with EDTA are collected. The blood samples are centrifuged at 1500 g for 10 minutes before plasma and whole blood are separated and frozen at -70 °C until analysis.

Table 2 summarizes numbers of CRC patients and controls included in papers I-IV. In papers I, II and IV the results from genotyping and Luminex analysis were compared with clinical covariates from the established CRC project data file.

Table 2: Number of patients and controls included in papers I-IV

Subjects	Paper I	Paper II	Paper III	Paper IV
CRC patients	370	381	60	174
Controls	316	618	-	-

Clinical CRC covariates used in the statistical analyses

Clinical covariates associated with CRC were collected from the patients' medical records available in the Cambio COSMIQ software (Cambio Healthcare Systems, Stockholm, Sweden), stored in the CRC project data file and used for statistical analyses. The clinical covariates used in the statistical analyses in papers I, II and IV are presented in Table 3.

Table 3: Clinical CRC covariates used in statistical analyses

Covariates	Defined as
Age ^a	Years
Gender	Man or female
Localization of tumour	Colon or rectum
Preoperative treatment	Yes or no
Radical operation	Yes or no
Mucinous cancer	Yes or no
Grade of differentiation	Low, medium or high
TNM stage	I, II, III or IV
Adjuvant treatment	Yes or no
Cancer recurrence	Yes or no
Total mortality ^b	Yes or no
Cancer-specific mortality ^c	Yes or no
Survival ^d	Years

a Age of the patient at the date of surgery

b Total mortality defined as death by other causes than CRC

c Cancer-specific mortality defined as death caused by CRC

d Survival based upon date of surgery and date of death or last follow-up

METHODS

Tissue homogenization

Tissue lysates were obtained from normal and tumour tissue biopsies that were thawed, cut into sufficient sizes, and put into RIPA buffer (containing 1XTBS, 1 % nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 0.004 % sodium azide and adding PMSF solution, sodium orthovanadate and protein inhibitor) (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) in an Eppendorf tube. The tissue biopsies were then homogenized and placed on ice for 30 min and centrifuged at 14000 g for 8 min at 4°C. The protein content of the lysate supernatant fluid was determined using the Bradford protein assay (Bio-Rad Laboratories, Ltd., Hertfordshire, UK), according to manufacturer's recommendations.

Immunohistochemistry

Embedding and sectioning services were performed by co-workers at the Pathology Laboratory, Department of Laboratory Medicine, Region Jönköping County.

Immunohistochemistry (IHC) was performed in papers I-III to visualize the expression and localization of the proteins of interest: CD93, PLA2G4C, PDGF-DD, PDGFR- β and phospho-specific platelet-derived growth factor receptor β (phPDGFR- β) in human matched tumour and normal tissue sections from CRC patients.

In papers I-III, epitope unmasking was performed using the antigen retrieval solution DIVA, and background staining was minimized by quenching the endogenous peroxidase activity with 3 % hydrogen peroxide.

Avidin and biotin detection

In papers I-III, IHC was performed using the avidin and biotin detection system. Non-specific binding between primary antibodies and human tissue samples was blocked either with normal goat or horse serum prior to incubation with primary antibodies at 4 °C overnight and secondary antibodies for 1 hour at room temperature. Detection of the antibody-antigen complex is based on the avidin and biotin complex followed by visualization with 3,3-diaminobenzidine tetrahydrochloride substrate. To give a contrast to the primary stain the sections were counterstained with hematoxylin before being dehydrated and mounted for light microscopy visualization.

Protein detection using the IHC method via antibody binding is a widely used method in clinical applications for diagnosis, prediction and prognosis (Coons, 1951). The formalin-fixed biological tissue samples are embedded shortly after surgery, which ensures that the tissue samples may be archived over time without changing their structure. The sectioning of the samples enables repeated investigations of staining of different proteins of interest. The IHC method enables identification and visualization of different proteins of interest as well as their location within the different layers of the tissue due to the binding of a specific antibody to an antigen. The binding is visualized either directly or indirectly by a fluorophore or a peroxidase-conjugated antibody.

Monoclonal antibodies are made from identical immune cells cloned from a parent cell, isolated by chromatography and bound to the same epitope. These antibodies are more difficult to produce and therefore more expensive than polyclonal antibodies. The polyclonal antibodies are a mixture of immunoglobulins that can bind to different epitopes of the target protein antigen of interest, resulting in a stronger signal. The monoclonal antibodies are more specific than the polyclonal antibodies. (MacPhee, 2010). There are many commercially available antibodies that are well described but even though the antibodies have a recommended dilution factor it may be necessary to perform tests of several dilutions before an optimal dilution factor for the tissue investigated is found. This process

may also require changes in the IHC protocols for optimized staining and may be a time-consuming process.

Western Blot

In papers I and III, Western Blot (WB) was used to reveal protein expression of CD93, PDGFR- β and phPDGFR- β . In paper I and III, CD93, PDGFR- β were measured in matched tumour and normal tissue lysates from CRC patients. In paper III, protein expression of phPDGFR- β was investigated in Caco-2 and HT-29 cell lysates.

The WB method consists of three parts: protein separation, transfer to a solid support, and visualization of the protein using a specific primary and secondary antibody. After denaturation of tissue lysates and cell lysates, proteins are separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) before they are transferred onto a polyvinylidene fluoride (PVDF) membrane followed by blocking of unspecific binding sites. A marker is also loaded onto the gel, which enables size determination of visual protein bands. A primary antibody specific to the protein of interest is added followed by incubation. Several steps of washing of the membrane after incubation ensure that only the bound protein of interest is left. A horseradish peroxidase (HRP) conjugated secondary antibody, specific to the primary antibody is added, followed by a new incubation period and washing of the membrane. The protein of interest is then visualized due to the oxidative reaction of luminol catalyzed by the HRP antibody and captured by a charged coupled device camera (Mahmood and Yang, 2012).

The WB method gives an opportunity to detect proteins of interest within biological samples such as tissue- and cell lysates, but since it demands several steps of manual handling it is time-consuming. The method is a semi-quantitative since it provides a relative comparison of protein levels and not the absolute measure of quantity. This is due to differences in both loading and transfer rates between the samples in separate lanes, which differ between separate blots and should therefore not be used to measure the concentration. Also, the signal generated during detection is not linear across the concentration range of samples.

Coomassie blue staining of the SDS-PAGE gel is an approach useful for control of loading since it ensure a semi-quantitative measurement of protein bands appearing on the gel. The PVDF membranes provide a proper mechanical support and allow the blots to be re-probed and stored minimizing the steps in the protocol and ensuring no unnecessary waste of samples. A disadvantage is that the background is higher in PVDF membranes compared to nitrocellulose membranes and careful washing is therefore important. However, leaving the membrane to wash for a long time may also reduce the signal of the bound antibodies (Mahmood and Yang, 2012).

Enzyme-linked immunosorbent assay

In papers I and III, commercially available Enzyme-linked immunosorbent assays (ELISAs), (R&D Systems Europe Ltd., Abingdon, UK) were used to quantify protein levels of CD93 and PDGF-DD in matched tissue lysates from CRC patients. In paper I protein levels of CD93 were also measured in plasma from CRC patients and controls. In paper III protein levels of MMP-1 and serpin family E member 1 (Serpin E1) were measured in cell supernatants from stimulated Caco-2 cancer cells.

The ELISA technique is a quantitative sandwich enzyme immunoassay that ensures binding of antigens to specific antibodies enabling detection or quantification of small amounts of antigens (e.g. proteins) in biological liquids such as plasma, serum, saliva, urine or cell culture supernatants. A commercial sandwich ELISA is a microtiter plate containing 96 wells pre-coated with a capture antibody. A sample which may contain the antigen of interest is added to the wells and if the antigen of interest is present in the sample it get bound by the immobilized antibody. After washing away of unbound substances, an enzyme-linked secondary antibody specific for the antigen of interest is added to the wells followed by a washing step to remove any unbound antibody-enzyme reagent. A substrate solution is then added to the wells and colour develops in the proportion to the amount of bound antigen of interest in the initial step. The colour development is stopped and the intensity of the colour is measured using a spectrophotometer. A standard of known concentration, which is

generated for each assay run, is then used to quantify the concentration of the antigen of interest (Hornbeck, 2015).

There are both advantages and disadvantages with the ELISA method. As mentioned, different types of biological samples can be analyzed and no radioactive substances are needed. The intra-assay precision within a commercial ELISA assay is quite accurate but the inter-assay precision may vary. The main advantage is the high sensitivity of the commercial ELISAs, and they also have a strong specificity for their coated capture antibodies. A disadvantage is that ELISAs often requires a large sample volume per well and each sample should be analyzed in duplicate. Also, only one analyte can be analyzed per sample, per ELISA assay (Fu et al., 2010) making it costly and time-consuming if more analytes need to be investigated. Variations in sample collection, processing and storage may cause sample value differences. The method includes several hands-on steps that may cause varying results due to poor precision.

Luminex

In paper IV, levels of inflammatory factors were measured in plasma from CRC patients by a commercially available Luminex assay, Bio-Plex Pro™ Human Chemokine Panel 40-Plex (Bio-Rad Laboratories, Inc., CA, USA), enabling analysis of 40 cytokines in 50 µl of plasma. The prognostic association between tertile levels of each cytokine, defined as low, medium or high levels, was analyzed in relation to survival defined as CRC-specific-, and total mortality.

Luminex is a multiplex bead-based sandwich immunoassay that combines ELISA and flow cytometry. The method shares the same immunological principle as ELISA with antibodies and antigens. The Luminex method using the Bio-Plex Pro™ Human Chemokine Panel 40-Plex involves the use of capture antibodies, which are coated onto magnetic beads (6.5 µm), filled with a specific mixture of fluorescent dyes. The colour-mixture of each bead is unique, enabling differently dyed beads coated with different antibodies to be mixed together in the same well. This permits the detection of multiple cytokines in a single sample.

After adding the coated magnetic beads to a 96-well microplate followed by incubation the magnetic beads are washed using a magnet underneath the plate in the automated washing station. This results in a magnetic field ensuring that the beads are not removed during the washing steps. In the next step, diluted samples and standards are added to the microplate followed by incubation allowing the capture antibody coupled to the beads to interact with the samples containing the target of interest. After incubation and washing to remove unbound materials, the biotin-conjugated detection antibodies are added, which are specific to a different epitope. This is followed by another incubation period creating a sandwich-complex. The final detection-complex is formed when streptavidin-phycoerythrin (streptavidin-PE) conjugate is added. PE serves as a fluorescent reporter (Figure 8) (Gupta V., 2014).

The beads are then read on a flow-based detection instrument with dual lasers. The red laser (635 nm) detects the bead, determining what cytokine is being measured, and the green laser (532 nm) assesses the PE fluorescence reporter intensity, which is proportional to the amount of bound cytokine (Gupta V., 2014). A digital processor manages the data output and the Bio-Plex Manager™ software presents data as median fluorescence intensity as well as the concentration (pg/mL). The concentration of analyte bound to each bead is proportional to the median fluorescence intensity of reporter signal (Bio-Rad Laboratories, 2017).

Figure 8: The principle of the Bio-Plex immunoassay modified from Bio-Plex Pro™ Human Chemokine assay instruction manual (Bio-Rad Laboratories, 2017).

An advantage of Luminex is that large numbers of cytokines can be detected simultaneously in a small volume of a single sample. This is possible due to the magnetic beads with difference in fluorescent dyes in combination with the wide range of detectable protein concentrations. When analysing numerous of cytokines the Luminex method is less time consuming than ELISA and more data can be gathered rapidly. The use of magnetic beads allows the method to be automated using a washing station and the magnetic separation ensures better reproducibility compared with vacuum filtration. To achieve proper results it is of great importance to vortex the magnetic beads properly before adding them to the plate since insufficient vortexing may result in lower concentrations of the cytokines measured. An inappropriate incubation time may also result in lower concentrations. Another disadvantage is that the Luminex analysis requires a specific instrument. For serum biomarker analysis the Bio-Plex assays together with MULTI ARRAY have been found to have the best performance, with the lowest limits of detection (Fu et al., 2010).

Caco-2 and HT-29 cell lines

In paper III the colon adenocarcinoma cell lines Caco-2 (Sigma-Aldrich, MO, USA) and HT-29 (Sigma-Aldrich) were used to study both the expression and proliferative effects of PDGF-D in CRC carcinogenesis *in vitro*. The Caco-2 cell line was also used in the gene array analysis to study effects of PDGF-D signaling on gene alterations.

The Caco-2- and the HT-29 cell lines are from human Caucasian colon adenocarcinomas where Caco-2 originates from a 72-year-old male while HT-29 originates from a 44-year-old female. Both cell lines are developed to emulate the human intestinal epithelium containing immortalized cells that rapidly can grow into confluent monolayers. Differentiated Caco-2 cells have been shown to express tyrosine kinase receptor of PDGF-D, PDGFR- β and c-Kit (Kaulfuss et al., 2013). As Caco-2 cells the HT-29 cells also express c-Kit (Bellone et al., 1997) but seem to express PDGFR- β more poorly compared to Caco-2 (Kaulfuss et al., 2013).

Human CRC model cell lines are common, preclinical model systems of the disease and have provided important insights into tumour- and cell biology, including the discovery of new anti-tumour compounds, and have revealed possible drug sensitivity, resistance and toxicity (Bracht et al., 2010, Weickhardt et al., 2012). The commercially available cancer cell lines are well defined and have a longer life-time than primary cell lines. It is easier to control experimental factors, and cell lines are widely used by investigators, thus facilitating comparison of results (Jones et al., 2008). Also, it is important to stress that cell line models in general are more efficient than animal models both in time and costs aspects. The Caco-2 cell line has been shown to share the same gene expression pattern involved in proliferation as seen in human colon cancer *in vivo* even though it lacks the tumour stroma, which is thought to affect processes such as proliferation in cancers, making it a suitable cell line for studying stimulatory effects affecting proliferation (Saaf et al., 2007).

A disadvantage is that cancer cell lines may be exposed to different environments such as sera, media and additional factors added to the media, as well as varying carbon dioxide

levels and temperatures, resulting in difference between data. Cancer cell lines derived from particular types of cancers are not representative of the clinical spectrum since in the clinic cancers are classified by stage and grade.

TACS® MTT cell proliferation assay

In paper III, the effects of recombinant PDGF-DD and imatinib stimulations on Caco-2 and HT-29 cell proliferation were assessed using the commercial available *in vitro* TACS® MTT cell proliferation assay (Trevigen, Inc., MD, USA).

The TACS® MTT cell proliferation assay is a colourimetric assay that may be assessed for cell viability, differentiation and proliferation among living cells. The assay is based on the reduction of yellow tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) to purple formazan by the mitochondrial NAD(P)H-dependent oxidoreductases and dehydrogenases that are present in live Caco-2 and HT-29 cancer cell lines (Figure 9). The reaction results in an intracellular purple formazan, which is quantified by spectrophotometric measurement at 550-600 nm. The rate of tetrazolium reduction is proportional to the rate of cell proliferation (Mosmann, 1983).

Figure 9: Principle of the MTT proliferation assay modified from TACS® MTT cell proliferation assays (Inc., 2009).

Tetrazolium salt assays such as MTT are high-throughput and useful in screening of cell lines for sensitivity to drug treatments and to changes in growth conditions when including growth factor, hormone and serum supplementation. The assay analysis is easy, does not include any steps of washing and many samples from different cell lines can be processed at the same time. The proliferation assay is also quantitative since there is a close correlation between viable cell numbers and the generated MTT-formazan signal, making it more efficient than counting viable cell manually with a hemocytometer (Mosmann, 1983). A limitation of the assay is that the linearity of the results may vary between MTT assays and between replicates of samples, which also was observed in our study. It has been shown that the MTT assay is less sensitive at cell numbers lower than 1000 cells per well (van Tonder et al., 2015). This problem was also observed in our study leading to cultivation of 2.5×10^4 Caco-2 cells and 1.5×10^4 HT-29 cells per well.

Nucleic acid extraction and reverse transcription

DNA was extracted from whole blood using a QiaAmp DNA blood kit (Qiagen, Hilden, Germany) and total RNA was extracted from matched tumour and normal tissue biopsies using the RNeasy kit (Qiagen), according to the manufacturer’s recommendations.

Total RNA (1 µg) from matched tumour and normal tissue biopsies from CRC patients or from Caco-2 cancer cells were reverse transcribed into complementary deoxyribonucleic acid (cDNA) with random primers and components of the High-capacity cDNA reverse transcription kit (Applied Biosystems Inc., CA, USA, according to manufacturer’s recommendations. Reverse transcription was performed at: 25 °C for 10 min; 37 °C for 1 hour; 85 °C for 5 min; 4 °C for ∞ in a Palm-Cycler™ (Corbett Life Science Pty. Ltd., Mortlake, Australia).

Polymerase Chain Reaction

In papers I and, II SNP analysis of *CD93* and *PLA2G4C* was done with TaqMan genotyping assays (Life Technologies, Ltd., Paisley, UK). In short, DNA (10 ng) was mixed with TaqMan Universal PCR Master mix II (Life Technologies) and TaqMan genotyping assays (Life Technologies) (Table 4) and amplified using the 7500 Fast Real-Time PCR system (Applied Biosystems).

Table 4: TaqMan genotyping assays (Life Technologies) used in papers I and II

Gene	SNP ID	Paper I	Paper II
<i>CD93</i>	rs2749812	x	
<i>CD93</i>	rs2749817	x	
<i>PLA2G4C</i>	rs1549637		x

rs: reference ID

In papers I and III, gene expression analysis was done by TaqMan gene expression assays using TaqMan probes on a 7500 Fast Real-Time PCR system (Applied Biosystems). In paper I, gene expression of *CD93* on cDNA from CRC tissues and matched normal tissues was

investigated. In paper III, gene expression of *cell adhesion molecule 5 (CEACAM5)*, *hyaluronan synthase 2 (HAS2)*, *lysyl oxidase (LOX)*, *MMP-1*, *Serpin E1*, *TNF super family member 4 (TNFSF4)* and *versican (VCAN)* was investigated from PDGF-DD stimulated and unstimulated Caco-2 cells to validate gene array results (Table 5). The gene expression results were normalized to the values of three reference genes; *human glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, *ribosomal protein lateral stalk subunit P0 (RPLP0)* and *TATA-box binding protein (TBP)* (Table 5). All gene expression assays in papers I and III contains exon-spanning probes reducing the risk of false positive amplification of any contaminating genomic DNA since the intron-containing genomic DNA sequence would not be amplified.

Table 5: TaqMan gene expression assays (Life Technologies) used in papers I and III

Gene	Assay ID	Paper I	Paper III
<i>CD93</i>	Hs00362607_m1	X	
<i>CEACAM5</i>	Hs00944025_m1		X
<i>GAPDH</i>	Hs02758991_g1	X	X
<i>HAS2</i>	Hs00193435_m1		X
<i>LOX</i>	Hs00942480_m1		X
<i>MMP-1</i>	Hs00899658_m1		X
<i>RPLP0</i>	Hs99999902_m1	X	X
<i>Serpin E1</i>	Hs00167155_m1		X
<i>TBP</i>	Hs00427620_m1	X	X
<i>TNFSF4</i>	Hs00182411_m1		X
<i>VCAN</i>	Hs00171642_m1		X

Hs: *Homo sapiens*; m1: the probe of the assay spans an exon junction and will not detect genomic DNA; g1: the assay may detect genomic DNA and the probe of the assay and primers may also be within a single exon.

Quantitative real-time polymerase chain reaction (qPCR) provides a sensitive, specific and reproducible detection and measurement of DNA products generated during each cycle of the qPCR (Heid et al., 1996, Ishmael and Stellato, 2008). Each cycle of the qPCR consists of three major steps. The denaturation-, annealing- and extension phase. During the denaturation phase of genotyping and gene expression analysis the genomic DNA is

denatured into separate strands. In the annealing phase the probe/primer hybridize to the complementary target sequence on the template DNA strand. The TaqMan probe have a fluorescent reporter dye on the 5'-end and a non-fluorescent quencher minor groove binder dye on the 3'-end. During the annealing phase the primer and TaqMan probe will hybridize to the target sequence if present. As long as the probe is intact the close proximity of the 3' quencher inhibits the fluorescent signal from the 5' reporter fluorochrome. The fluorescent signal is emitted when the probe is cleaved. In the extension phase the 5' to 3' exonuclease activity of the thermostable Ampli *Thermus aquaticus* (Taq)-Gold® DNA polymerase cleaves the hybridized probe, releasing the reporter dye from the quencher. The fluorescence signal generated by qPCR amplification indicates which alleles are present in the sample in the genotyping analysis. During each cycle of qPCR the exponential signal is proportional to the amplified material obtained during gene expression analysis (Scientific, 2017b, Scientific, 2017a, Holland et al., 1991). During the cycles of the qPCR the exponential increase in fluorescent signal is proportional to the amplified material. This makes it possible to quantify the amount of starting material by relating it to a standard curve.

The use of TaqMan genotyping and TaqMan gene expression assays ensures an effective analysis. Since the assays contain minor groove binders a high specificity for the target site is ensured. TaqMan gene expression analysis by qPCR is a valuable tool for validation of gene array results (Ishmael and Stellato, 2008). On the other hand, for gene expression analysis the high sensitivity of qPCR also requires accurate and precise pipetting, high-quality RNA, an accurate estimation of the RNA concentration as well as an efficient reverse transcription. Variations in these technical parameters may influence the accuracy and precision of the results (Remans et al., 2014). Another limitation to gene expression analysis is to choose proper reference genes for normalization and the need to run standard curves for each gene of interest with each assay (Ishmael and Stellato, 2008).

Affymetrix® GeneChip® Whole Transcript Expression array

In paper III, the Affymetrix® Genechip® Whole Transcript Expression array (Affymetrix, CA, USA) was used to evaluate the effects of PDGF-D on gene expressions using the Caco-2 cancer cell line as a model. In this paper, employees at the department of Genome Analytics at the Helmholtz Centre for Infection Research, Braunschweig, Germany, helped to perform gene array analysis and subsequent interpretation of data.

Gene arrays, also named microarrays, are solid supports where upon a large collection of gene specific nucleic acids are situated at defined positions. During the gene array analysis double-stranded cDNA is synthesized from RNA samples. Then an *in vitro* transcription, including a biotin-labelled nucleotide, is performed producing biotin labelled complementary RNA (cRNA) from the cDNA. The cRNA is then purified to remove unincorporated nucleotides, inorganic phosphates and salt before it is fragmented and hybridized to the Affymetrix® GeneChip® array. The hybridized gene array is then stained with PE-conjugated streptavidin and scanned (Figure 10). The emitted light is proportional to the amount of bound target sequence and hence the gene expression level (Corporation, 2011, Inc., 2009-2010). The analysis of the obtained gene array data after scanning is performed using GeneSpring 11.5.1 (Agilent Technologies, Inc., CA, USA), which provides analysis of changes in gene expression (Agilent Technologies, 2011).

A major advantage of gene array analysis is that the method can provide information on thousands of targets in a single experiment. The GeneSpring software is helpful in both visualization of data and in comparing results from different data sets. The Affymetrix® Genechip® Whole Transcript Expression array ensures expression analysis over the entire transcript since the probe selection region is defined by exons. This allows identification of alternatively spliced transcripts. Each spot on the chip contains a certain number of copies per gene and if a sample contains a higher concentration of certain genes it may lead to a saturation of the spot leading to measurement of too low RNA levels.

Figure 10: Schematic overview of the gene array process modified from GeneChip® WT terminal labeling and hybridization user manual (Inc., 2009-2010).

Statistical analyses

Papers I and II: The Chi squared test was used to test the genotype frequencies for deviations from the Hardy Weinberg equilibrium for all SNPs analyzed.

Paper I, paper II and paper IV: To evaluate differences in the frequencies of the SNPs analyzed between patients and controls as well as the distribution of clinical covariates (papers I and II), and to determine the correlation between CRC-specific mortality (paper IV) and the clinical covariates within the patient group a chi-squared test (Pearson's chi-squared test) was performed.

Papers I-IV: Differences in protein expression levels between tumour and matched normal tissues in relation to clinical covariates (papers I and III), differences in plasma protein levels between patients and controls in relation to clinical covariates (paper I), differences between clinical covariates among stage II CRC patients (paper II) and differences in levels of inflammatory factors between TNM stage combinations in relation to clinical covariates, (paper IV) were examined by the Wilcoxon signed-rank test (also named the Wilcoxon rank-sum test or Mann-Whitney *U* test).

Papers I, II and IV: A Shapiro-Wilk test was used to determine the normal distribution. Cancer-specific- and disease-free survival were analyzed using the Kaplan-Meier analysis and log-rank test. The Cox regression analysis was performed for univariate- and multivariate analysis in association with clinical covariates (papers I and II). Analysis of CRC-specific- and total mortality was performed with Kaplan-Meier analysis and a log-rank test. Both a univariate and a multivariate Cox regression analysis were performed to investigate unadjusted and adjusted effects on the inflammatory tertile levels in relation to either CRC-specific- or total mortality in association with clinical covariates (paper IV). The goodness of fit of the multivariable models was tested by Harrell's C index. (papers I, II and IV).

Paper III: The treatment effects on cancer cell lines in relation to cell proliferation were examined using a One-Way ANOVA with Post Hoc Multiple Comparisons (Bonferroni) test. For the gene array results signal intensities (raw data) were log2 transformed and normalized using the robust multichip average. Gene expression values for each condition were given as a relative expression to the mean expression value calculated from the intensities of all conditions (mean centralization, normalized data). To determine differentially expressed genes after stimulation a statistical t-test followed by a statistical error correction (Benjamini-Hochberg) were applied to log2 normalized gene expression data ($n = 3$). Genes with corrected p -values < 0.05 were considered as differentially expressed.

Statistical analyses were performed in IBM® SPSS® Statistics, 2012 (version 19 and 21, SPSS Inc., IL, USA) or STATA 12 and 13 software (StataCorp, Stata Statistical Software; release 12 and 13, College Station, TX, USA).

In all studies, p -values < 0.05 were considered statistically significant, where * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** and indicates $p < 0.001$.

ETHICS

The CRC project was approved by the Local Ethics Committee of Linköping University, Sweden, (approval number 98113 and 2013/271-31) approving collection of blood samples, matched control and tumour biopsies, as well as the collection of clinical covariates associated with CRC from the patients' medical records available in the Cambio COSMIQ software (Cambio Healthcare Systems). The patients were de-identified and the samples were collected together with routine samples not causing the patients an extra burden during sampling.

RESULTS AND DISCUSSION

CD93 gene polymorphism is associated with disseminated colorectal cancer (Paper I)

CD93 has been studied in various types of diseases, including CVD, as well as in various cancer forms but not in CRC prior this study. Since the initially aim of the thesis was to study possible associations of underlying CVDs on CRC disease and prognosis the aim of paper I was to study the expression and localization of CD93 in normal and adjacent tumour tissues and CD93 concentrations in plasma from controls and CRC patients. The association of two SNPs of *CD93*, rs2749812 and rs2749817 not found to be in linkage disequilibrium (LD) to another, were also studied (Malarstig et al., 2011). The SNP rs2749812 has previously been associated with risk of myocardial infarction (Malarstig et al., 2011) and coronary heart disease (van der Net et al., 2008), while SNP rs2749817 has been associated with the risk of breast cancer (Lee et al., 2009).

An increased expression of CD93 was seen in vascular endothelial cells around blood vessels in the cancerous tissues compared to expression observed in the same locations in matched normal tissues. The same expression and localization patterns have been seen in other types of cancers such as in glioblastoma (Langenkamp et al., 2015) and nasopharyngeal carcinoma (Bao et al., 2016). The production of CD93 has been shown to be mediated by inflammatory factors such as MMPs and TNF- α (Bohlson et al., 2005). The production of CD93 may also be induced by other unknown inflammatory factors in vascular endothelial cells.

Langenkamp *et al.* investigated the function of CD93 in tumour angiogenesis and endothelial cell biology and showed that knock-down of CD93 inhibited adhesion and migration of endothelial cells as well as tube formation *in vitro* due to defects in cytoskeletal rearrangement by the interaction of the C-terminal domain of CD93 with myosin and loss of endothelial adherence junctions. These results led to the conclusion that CD93 was essential in establishing endothelial cell-cell and cell-matrix contacts as well as migration and tubular

morphogenesis, suggesting that CD93 promotes the formation of well-functioning tumour vessels (Langenkamp et al., 2015). It is possible to assume that the same actions may also appear in CRC, which would explain the increased expression of CD93 in vascular endothelial cells in blood vessels.

Since expression and localization of the CD93 protein was seen in CRC tissue, the protein levels of CD93 were further investigated in matched tissue lysates as well as in plasma. Comparing tissue protein levels of total CD93 (cell bound and soluble form of CD93) an 82 % higher level was seen in CRC tissue lysates compared to matched normal tissue lysates. This result may be due to an ongoing angiogenesis and increased inflammation in the CRC tissue. This assumption is strengthened by the findings of Kao *et al.* showing that the EGF-like domain of CD93 is important in the angiogenetic process since binding of EGF to its EGF receptor induces cellular signalling triggering the development of angiogenesis (Kao et al., 2012).

Even though an increased expression of CD93 was observed in both CRC tissue sections and CRC tissue lysates the gene expression analysis did not reveal any differences in *CD93* expression between CRC tissue and matched normal tissue levels of CD93. In this case it is important to remember that divergence between protein and messenger RNA (mRNA) expression is a common finding (Schwanhaussner et al., 2011). Also a possible pre-existing inflammatory condition within the normal tissue distant from the location of the CRC tumour could explain why there was no difference in *CD93* expression levels in CRC tissues compared to the matched normal tissues. This was suggested as a possible explanation in a different study by Mounier *et al.* where similar results in gene expression were observed (Mounier et al., 2008).

In contrast to the increased levels of total CD93, a 30 % lower level of the soluble form of CD93 was detected in plasma among CRC patients compared to healthy controls. This result was quite surprising since the soluble form of CD93 has been shown to be cleaved by MMPs normally found in higher levels in CRC. TNF- α has also been shown to contribute in the releasement of CD93 (Bohlson et al., 2005). In a study by Greenlee *et al.* the soluble CD93 was shown to function as a bridging molecule between apoptotic cells and macrophages

ensuring efficient engulfment of apoptotic cells. Also, Greenlee *et al.* suggested that proteases such as MMPs responsible for the cleaving and release of CD93 were specific to activated macrophages, meaning that the MMPs lose their ability to produce the soluble form of CD93 when the macrophages are quiescent (Greenlee et al., 2009). Endothelial cells also express soluble CD93 and serve as an additional source of the soluble form (Fonseca et al., 2001), which might explain the lower levels observed in CRC plasma if macrophages and their contributing proteases go quiescent due to unknown immunologic imbalances in CRC patients. The presence of tissue inhibitors of metalloproteinases secreted by cells may also inhibit the function and decrease the expression of MMPs (King and Robins, 2006a) and may therefore be another important factor in explaining the lower levels of soluble CD93 in plasma.

Another observation was that approximately 66 % of the CRC patients had plasma levels of CD93 below 142 ng/mL, which has been suggested as a threshold associated with increased risk of myocardial infarction among Swedish patients with coronary artery disease (Malarstig et al., 2011). Since no clinical information regarding possible underlying CVDs was available no conclusion on whether low plasma levels of CD93 might indicate a risk of developing both CRC and CVD, or if the diseases may reflect similar physiological and pathological mechanisms in the regulation of CD93 could be drawn.

Two SNPs of *CD93*, rs2749812 and rs2749817, previously associated with risk of CVD and breast cancer, were investigated to reveal their potential associations with CRC. For both SNPs no differences in genotype distribution among CRC patients and controls were observed. This indicates that none of the SNPs are risk factors for contracting CRC. Among the patients, an adjustment for localization, gender and age did not affect the results.

The genotype distribution of rs2749817 showed an increased frequency of the T/T genotype in stage IV compared to stage I-III. By studying the T/T genotype in relation to CRC-specific survival it was shown to be associated with worse prognosis and a lower recurrence-free survival. It was also associated with an increased risk of disease recurrence among CRC patients that were tumour-free after surgery compared to patients carrying the C/C- or the C/T genotype. When comparing the genotype frequencies of rs2749817 to the tissue levels

of total CD93, tumour tissue levels among CRC patients carrying the T/T genotype were 20 % higher compared with the levels in patients carrying the other genotypes. This suggests that the T/T genotype of rs2749817 may affect the expression of CD93 in CRC tissue.

The association of the T/T genotype of rs2749817 with disseminated cancer at diagnosis and an increased risk of recurrence after radical operation suggests a lower resistance to the dissemination of tumour cells as well as a reduced ability to control metastasis. The exact mechanism of the T/T genotype of rs2749817 is still unknown but it seems to be associated with increased tissue level of CD93 in CRC which might affect angiogenesis, growth and spread of CRC. This SNP is situated downstream of the 3' untranslated region of *CD93*, in an intergenic region consisting of long non-coding RNAs. Long non-coding RNAs may alter their activity by folding into structures enabling them to interact with transcription factors affecting the transcription of nearby genes (Ling et al., 2015). This may influence the translation and post-translational modifications of *CD93*. Also, the role of the T/T genotype of rs2749817 on survival remains to be further investigated, but the results in paper I suggest that the SNP is a potential risk factor for predicting survival among CRC patients.

Since the CRC patients studied in paper I had a high mean age there might be a risk of other underlying inflammatory disorders affecting the CD93 levels in plasma or in tissue lysates. The lack of clinical data regarding possible underlying disorders such as CVDs at the time of the study is a clear limitation and no conclusions can be made regarding their possible impact on CD93 levels.

In summary, the results of paper I show that CD93 had an increased expression in CRC tissue, which may reflect the ability of CD93 to promote formation of well-functioning tumour vessels as well as an ongoing angiogenesis and inflammation. The lower plasma levels of CD93 in CRC patients may be a result of the lost ability of MMPs to produce the soluble form of CD93 when the macrophages are quiescent leaving endothelial cells as the only source capable of producing soluble CD93. Most importantly, the T/T genotype of SNP rs2749817 of *CD93* may be associated with disseminated CRC at diagnosis and an increased recurrence rate after radical operation. This indicates that the T/T genotype of SNP rs2749817 may be a potential marker related to severe tumour disease and spread, even at the time of diagnosis.

Prognostic significance of PLA2G4C gene polymorphism in patients with stage II colorectal cancer (Paper II)

Several studies have shown expression of cytosolic PLA2 enzymes in CRC (Dimberg et al., 1998, Osterstrom et al., 2002) and the expression of PLA2G4C has been suggested to be involved in breast cancer cell chemotaxis and invasion (Tian et al., 2011). The cellular source, localization or possible clinical association of PLA2G4C in CRC has not previously been described. In paper II, the focus was therefore to study the expression and localization of PLA2G4C in CRC tissue and to study the association of the *PLA2G4C* SNP, rs1549637, as a potential risk factor for CRC and a predictor of disease outcome among CRC patients.

Expression of PLA2G4C was mainly localized in epithelial cells in both tumour and matched normal tissue from CRC patients with an equal expression and was in line with previous studies (Panel et al., 2006, Yoo et al., 2011, Osterstrom et al., 2002).

SNP rs1549637 analysis revealed no differences in either the genotypic or in the allelic frequencies among CRC patients and controls. This observation indicates that the SNP rs1549637 is not to a risk factor for contracting CRC. Among CRC patients the Kaplan-Meier analysis showed a significant difference in CRC-specific survival between the genotypes of rs1549637 among CRC patients of all TNM stages. After combining the A/A and the A/T genotype due to low prevalence of the A/A genotype, survival analysis revealed that carriers of the A allele had the highest risk of CRC-specific mortality compared to patients carrying the T allele. The A allele remained an independent predictor of increased CRC-specific mortality even after adjustment for the clinical covariates; age, gender, TNM stage, tumour localization, adjuvant treatment, T4 tumour invasion, histopathological type and differentiation. Also, compared with T allele carriers the CRC patients carrying the A allele had a poor prognosis in stage II CRC, with an increased risk of CRC-specific mortality even after adjustment for the mentioned clinical covariates.

Stage II disease is found in approximately 30 % of all CRC patients (Jemal et al., 2004) and even though stage II disease has in general a good prognosis 20-25 % of CRC patients will still experience recurrence of the cancer (Sobrero, 2006, O'Connor et al., 2011). Adjuvant

treatment has been shown to be beneficial in stage III CRC (Bockelman et al., 2015), but it remains controversial in stage II CRC (Sobrero, 2006, O'Connor et al., 2011, Glimelius, 2015). To be able to identify and select these subgroups of CRC patients for adjuvant treatment the currently used markers are: elevated CEA levels, lymphovascular or perineural invasion, perforation, poorly differentiated tumour, T4 growth, and removal of fewer than 12 lymph nodes from the specimen. However, all these markers are associated with weak prognostic characteristics (Bockelman et al., 2015) underlining the importance of finding risk factors with better prognostic characteristics. In a study by Buhmeida *et al.* another PLA2G4 member, PLA2G4A, was suggested as a prognostic marker for the selection of patients at high risk of recurrence in stage II CRC that might benefit from adjuvant treatment. They found that PLA2G4A-positive staining of tumours was associated with lower CRC-specific survival. On the other hand, no associations with conventional risk factors such as age, gender, depth of invasion or lymph node status were found (Buhmeida et al., 2009).

The exact function of the A allele of the SNP rs1549637 are currently unknown. The SNP is situated in the 3' untranslated region of *PLA2G4C*, which may alter the mRNA level or even modify the transcriptional activity of the gene (Kao et al., 2000).

There are some limitations to this study that are important to consider in further studies of PLA2G4C. First, no gene expression analysis was performed, which may have given further insights on expression of *PLA2G4C* in matched tumour and normal tissues from CRC patients. Second, there was no investigation performed whether the SNP rs1549637 was in LD with another SNP of *PLA2G4C*, which may be the actual SNP associated with the clinical outcome among the CRC patients. Third, risk factors such as CEA as well as vascular, lymphatic and neural tumour invasion were missing among CRC patients in the oldest part of the study period and were therefore not included in the analyses.

In summary, PLA2G4C was shown to be expressed in epithelial cells in both CRC tissue and matched normal tissue. The SNP rs1549637 of *PLA2G4C* seem not to be a risk factor for contracting CRC. On the other hand, the A allele of this SNP was shown to be a potentially stronger predictor for CRC-specific mortality in stage II than the available conventional risk

factors used in the clinical practice for selection of stage II patients for adjuvant treatment. The A allele of SNP rs1549637 may be a potential marker for finding stage II CRC patients that would benefit from a more comprehensive follow-up and adjuvant treatment.

Possible role and therapeutic target of PDGF-D signalling in colorectal cancer (Paper III)

The production of various growth factors and cytokines are important for cancer cells to enable progressive growth and development of cancer. PDGF-D is expressed in various types of cancers such as breast cancer and CRC (Ahmad et al., 2011, Chen et al., 2016) and is shown to be involved in the regulation of cellular processes important in cancer development (Ustach and Kim, 2005, Li et al., 2003, Chen et al., 2016). PDGF-D induces its cellular effects by binding to and activating the receptor PDGFR- β (Li et al., 2003, Wang et al., 2009), a tyrosine kinase receptor, which, due to its phosphorylation is able to trigger a number of signalling pathways also in CRC (Chen et al., 2016). Still, little is known regarding PDGF-D's possible role in CRC. In this study the expression and localization of PDGF-D in CRC tissue were studied as well as the possible involvement of PDGF-D signalling in colorectal carcinogenesis *in vitro*.

The expression and localization of both PDGF-D and its receptor, PDGFR- β , were studied in sections from CRC tumour tissue and matched normal tissue. PDGF-D was mainly found in the vascular endothelial cells of blood vessels situated in the cancerous microenvironment and in the matched normal tissue, which was in line with a previous study (Chen et al., 2016). In the study by Chen *et al.* the expression of PDGF-D was associated with clinicopathological features such as TNM stage, lymph node invasion, and tumour differentiation (Chen et al., 2016). In this study the correlation between expression levels of PDGF-D and clinicopathological covariates was not investigated since no differences in tissue expression levels of the protein were observed by ELISA. The PDGFR- β receptor was mainly located in both normal epithelial cells and in tumour-associated stromal cells of the cancerous microenvironment, which was in line with previous reports (Sundberg et al., 1993, Lindmark et al., 1993). The activated form of the receptor, pPDGFR- β , was observed in normal epithelial cells and stromal cancerous tissue. The inactivated and the activated form of PDGFR- β were observed in cell lysates from the CRC cell lines Caco-2 and HT-29. These observations led to the assumption that both PDGF-D and its receptor, PDGFR- β , may be

involved in the carcinogenic progress of CRC and led to further investigation of potential genetic effects induced by PDGF-D signalling *in vitro*.

The Caco-2 cell line was used as a model to reveal possible genetic effects after 24-hour stimulation with PDGF-D followed by a gene array analysis. The PDGF-D stimulation induced an alteration of target genes, which may potentially modulate the tumourigenic activities of cancer cells, such as ECM integrity, angiogenesis, migration, metastasis and proliferation to promote further CRC progression. These results imply that PDGF-D signalling may have an important role in CRC development and progression.

PDGF-D has previously been shown to be important in the EMT process that is involved in the development of metastasis, in which epithelial cells are transformed into mesenchymal cells due to morphologic changes (Wu et al., 2013, Chen et al., 2016). To ensure migration and invasion the mesenchymal cells may increase the activity of proteases such as serine proteases and MMPs, which ensures a path for the migration and invasion of the mesenchymal cells through the ECM (Wu et al., 2013). The gene expression pattern induced by PDGF-D revealed an increased expression of serine proteases, MMPs, collagens integrins and selectins that are important for the detachment of and migration of tumour cells during metastasis. Integrins together with selectins also helps the cancer cells to avoid destruction of immune cells in the blood stream. Also, collagen, laminin and fibronectin were upregulated and are involved in the attachment of cancer cells in the blood stream (King and Robins, 2006a, Alizadeh et al., 2014) strengthening the indication of PDGF-D's involvement in EMT but also in the process of angiogenesis and metastasis, which are important processes in cancer development and progression.

PDGF-D clearly plays an important role in development and progression in several cancers by deregulation of various cellular processes. Therefore, targeting PDGF-D might be an important strategy for treatment in CRC. Imatinib, an ATP-competitive tyrosine kinase inhibitor which is able to inhibit PDGFR- β phosphorylation and activation, has been shown to

inhibit both growth and invasion in a CRC cell model (Kitadai et al., 2006). It is also a well-tolerated drug with mild to moderate clinical side effects (Demetri et al., 2002) and has shown promising results in management of metastatic CRC (Kelley et al., 2013, Hoehler et al., 2013, Al-Batran et al., 2007). Due to its promising effects on metastatic CRC, its less severe side effects and its ability to inhibit PDGFR- β the potential effects of imatinib on CRC proliferation and PDGF-D signalling were investigated.

Expression of phPDGFR- β was investigated in cell lysates from the Caco-2- and HT-29 cell line, stimulated with imatinib, imatinib and PDGF-DD or PDGF-DD only. PDGF-DD stimulation induced phPDGFR- β expression and imatinib attenuated the basal phPDGFR- β expression. An increased proliferation was induced in Caco-2 and HT-29 cancer cells stimulated with PDGF-DD suggesting that the binding of PDGF-D to its PDGFR- β resulted in a phosphorylation of the receptor and a further downstream signalling triggering the proliferation (Bergsten et al., 2001). Stimulation with imatinib gave a decreased proliferation in unstimulated and in PDGF-DD stimulated cells. The observed inhibition on proliferation caused by the tyrosine kinase inhibitory effects of imatinib may be a result of inhibition of both the PDGFR- β as well as c-Kit, which also is expressed in both cell lines (Kaulfuss et al., 2013, Bellone et al., 1997), leading to the anti-proliferative effects (Kitadai et al., 2006).

Cancer cells express peroxisome proliferator-activated receptors (PPARs) initially thought to have anti-inflammatory and anti-tumourigenic activities (Rohrl et al., 2011). In CRC the receptor activity has been shown to be lost due to mutation (Sarraf et al., 1999) or the activation of the receptor is reduced because of down-regulation of its cellular ligands (Shureiqi et al., 2000) leading to further tumour progression. PPARs have also been shown to mediate angiogenesis in CRC by inducing VEGF in HT-29 cells indicating that the presence of PPAR agonists may facilitate angiogenesis (Rohrl et al., 2011). This is still debated in the literature and need to be further evaluated before any firm conclusions can be drawn.

There are some limitations to this study. PDGF-D stimulations *in vitro* do not reflect the complete carcinogenic picture appearing in CRC patients because of individual differences. *In vitro* studies are suitable for studying possible effects upon carcinogenesis due to stimulation and may give a hint of how the actual carcinogenic regulation works. In this study any possible regulation of c-Kit or PPARs influenced by imatinib stimulation were not further investigated.

In summary, paper III shows that PDGF-D and its receptor, PDGFR- β , are expressed in CRC. Stimulation of CRC cells with PDGF-D results in expression of genes of importance in CRC carcinogenesis. Thus, PDGF-D/PDGFR- β signalling may be an important pathway in CRC progression and a target in CRC treatment.

Circulating inflammatory factors associated with worse long-term prognosis in colorectal cancer (Paper IV)

CRC develops over time through the accumulation of genetic mutations and epigenetic silencing of tumour suppressor genes but the interaction between a tumour and the tumour microenvironment is also important (Itatani et al., 2016). Inflammatory factors such as cytokines are similar to growth factors, which can bind to receptors present on cells appearing in both the innate- and adaptive immunity, enabling infiltration of inflammatory cells that may contribute to cancer development (Mager et al., 2016). In this study, a possible association between the concentrations of several cytokines in plasma at the time of CRC surgery and CRC prognosis was investigated.

Plasma levels were measured in 174 CRC patients by a commercially available Luminex kit that detects and quantifies 40 cytokines. Since median plasma levels (pg/mL) of all cytokines included did not vary between stage combinations (TNM stages I and II vs. TNM stages III and IV) each factor was divided into three tertile levels: low, medium, or high. Cytokine levels were then statistically evaluated in relation to the survival time and clinical covariates of the CRC patients. The clinical covariates were radical surgery, TNM stage, preoperative- and adjuvant treatment. Since age was highly associated with the increased risk of death in general this covariate was also included.

In total, 40 of the 174 CRC patients had died due to CRC (mainly stage III or IV patients (~88 %)), while 26 patients died for other reasons, and 108 patients were still alive at follow-up. Kaplan-Meier analysis showed an increased risk for both total- and CRC-specific mortality for the highest tertile levels of the cytokines. For most of the cytokines an association between higher levels and increased total- or CRC-specific mortality appeared two years after surgery.

To study the potential relation between the tertile levels of the cytokines and total mortality a univariate Cox regression analysis was performed in which single variables, cytokines and

clinical covariates separately were taken into account in relation to total mortality. The highest tertile levels of 14 cytokines including chemokine C-C motif ligand (CCL) 1, CCL3, CCL15, CCL20, C-X3-C motif chemokine ligand 1 (CX3CL1), Chemokine C-X-C motif ligand (CXCL) 1, CXCL10, CXCL13, IFN- γ , IL-1 β , IL-2, IL-4, IL-8/CXCL8 and IL-10 were all statistically associated with risk of total mortality. Only nine of the cytokines including CCL1, CCL15, CCL20, CX3CL1, CXCL13, IFN- γ , IL-2, IL-4, and IL-10 remained significantly associated with total mortality after adjustments for age, radical surgery, TNM stage, preoperative- and adjuvant treatment. Since univariate Cox regression analysis only compares one variable at a time in relation to total mortality and does not give a clear picture of the effects of potential interactions between the levels of cytokines, clinical covariates and total mortality a multivariate Cox regression analysis was also performed. In the multivariate Cox regression analysis, the levels of all cytokines and clinical covariates were considered in association with total mortality. None of the nine inflammatory factors remained significant for total mortality.

The relation between tertile levels of the cytokines and CRC-specific mortality was also investigated. The univariate Cox regression analysis showed that the highest tertile levels of eight cytokines including CCL1, CCL3, CCL15, CCL20, CX3CL1, CXCL16, IL-4 and IL-8/CXCL8 were associated with CRC-specific mortality. After adjustment for the clinical covariates, only four cytokines, CCL1, CCL20, CX3CL1 and IL-4, remained while two new factors, CCL24 and TNF- α , also became significant for CRC-specific mortality. In previous studies CCL20, CCL24, CX3CL1, IL-4 and TNF- α have been grouped as pro-tumourigenic cytokines in CRC (Itatani et al., 2016, Mager et al., 2016). This indicates that these cytokines may play a potential important role in promoting CRC progression. In the multivariate Cox regression analysis only CCL1 and CCL24 remained associated with CRC-specific mortality together with TNM stage IV and radical surgery, which both are strong predictors for diagnosis and treatment in CRC.

CCL1 is secreted from fibroblasts (Yeh et al., 2015) and Th2 cells (Zingoni et al., 1998). It has been associated with other types of cancers (Yeh et al., 2015) but little is known about its effects in CRC. The Th2 cells express the C-C motif chemokine receptor (CCR) 8 receptor (Rivino et al., 2004), which is activated by CCL1 (Roos et al., 1997). CCR8 also mediates the recruitment of Th2 cells to sites of inflammation (Chensue et al., 2001). The CCL1-CCR8 autocrine loop has been shown to have a protective function in cancer by enabling lymphoma and T cell leukaemia cells to avoid apoptosis *in vitro* (Van Snick et al., 1996, Ruckes et al., 2001) and to be involved in T cell transformation (Tamguney et al., 2004). In this study, higher tertile levels of CCL1 were associated with a 2.8 fold increased risk of CRC-specific mortality and it seems likely that the CCL1-CCR8 autocrine loop might help the CRC cells to progress their development and spread by avoiding destruction by immune cells. This study indicates that CCL1 may be an important inflammatory factor in CRC.

CCL24 attracts and activates both eosinophils and basophils via its receptor, CCR3, during Th2 cell responses (Forssmann et al., 1997) and was in this study associated with a 2.5 fold increased risk for CRC-specific mortality. In a study by Cheadle *et al.* expression of CCL24 was especially seen in both primary and metastatic CRC and to a lower degree in the surrounding non-neoplastic tissue. They also showed that IL-4 and IL-13 could stimulate overexpression of CCL24 in CRC cells. This expression of CCL24 was strongly associated with poor prognosis among CRC patients (Cheadle et al., 2007). In this study, high tertile levels of both IL-4 and CCL24 were observed and associated with CRC-specific mortality but did not remain for IL-4 after the multivariate Cox regression analysis. No conclusions regarding the possible interaction between IL-4 and CCL24 can be drawn in this study.

Even though cytokines analyzed in this study may be associated with a worse prognosis over time there are studies showing associations with high levels of inflammatory factors and lower survival in CRC, but also among CRC patients with good prognosis as well as in healthy individuals (Mansouri et al., 2016, McSorley et al., 2016). These observations may indicate that our finding of a worse prognosis in association with increased levels of inflammation is a

general phenomenon and not only related to the CRC disease. It may reflect an expression of a fragile host.

There are limitations that need to be taken into consideration in this investigation. First, a healthy control group of patients without CRC was not included since the focus of the study was survival among CRC patients only. Therefore, no conclusions regarding possible differences or associations between patients and healthy individuals in levels of the inflammatory factors studied can be drawn. However, there are several previous studies that investigated differences in cytokine levels among patients and healthy controls (Hillenbrand et al., 2012, Thorsen et al., 2013). Second, it is important to be aware that the level of cytokines released during CRC carcinogenesis may also be affected by other underlying diseases or by systemic inflammation in general. Since no clinical data regarding this matter was available, these possible associations cannot be conclusively stated.

In summary, a correlation between higher tertile levels of cytokines and an increased mortality among the CRC patients in general appeared two years after surgery. High tertile levels of several cytokines at time of surgery were in this study associated with worse long-term prognosis in CRC patients, both expressed as total mortality and CRC-specific mortality. This may indicate that the increased inflammatory levels may not only be due to the CRC disease but inflammation in general that lead to an expression of a fragile host. Higher levels of CCL1 and CCL24 were the only factors that remained significant for CRC-specific mortality after multivariate adjustment and may be important inflammatory factors for further investigation.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The aim of this thesis was to study associations of both circulating and genetic factors with CRC disease progression to gain further knowledge on the complex nature of CRC. The increased knowledge of the factors contribution in CRC progression and prognosis will hopefully contribute to an early CRC diagnosis, better treatment strategies and follow-up of CRC patients. The main findings in this thesis and their potential role in CRC progression are summarized in Figure 11.

Figure 11: Summary of the main findings in paper I-IV.

A SNP of *CD93* correlated with an increased expression of CD93 in CRC tumour tissue, a risk of disseminated CRC at diagnosis and a risk of CRC recurrence after surgery. In addition, a SNP of *PLA2G4C* was a potentially stronger predictor for CRC-specific mortality in stage II than the available conventional risk factors used in the clinical practice for selection of stage II patients for adjuvant treatment. These SNPs should be further validated in larger and unrelated CRC cohorts to establish their role in clinical practice to predict prognosis. Furthermore, clinical treatment studies could reveal new insights into their potential to personalize treatment. The increased expression of CD93 in CRC tissue related to the *CD93* SNP should also be further evaluated as a possible histological marker for prediction of prognosis.

PDGF-D signalling affected the alteration of genes important in CRC progression *in vitro* and stimulation with imatinib dampened PDGF-D induced proliferation. To gain further knowledge on PDGF-D signalling in CRC progression *in vivo* studies in animal models would be of importance. As imatinib is an established therapeutic drug on the market a clinical study could be taken into consideration to evaluate the therapeutic potential on CRC progression in man.

High levels of CCL1 and CCL24 in plasma taken at surgery correlated with an increased risk of CRC-specific mortality. These factors should be further evaluated in a larger and independent CRC cohort to reveal their possible importance in prognosis prediction. Comparing longitudinal levels of CCL1 and CCL24 pre- and post-surgery could reveal insights into their possible role as markers for recurrence of disease. These factors would possibly minimize the need for follow-up with methods that are cumbersome, costly or with high burden for patients such as colonoscopy, computed tomography and high resolution magnetic resonance imaging.

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