Stromal components and micro-RNAs as biomarkers in pancreatic cancer

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Till Johanna
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ABSTRACT

Background
Pancreatic ductal adenocarcinoma (PDAC) patients have the poorest 5-year survival rates of all cancer forms. It is difficult to diagnose at early disease stages, tumour relapse after surgery is common, and current chemotherapies are ineffective. Carbohydrate antigen 19-9 (Ca 19-9), the only clinically implemented PDAC biomarker, is insufficient for diagnostic and screening purposes.

PDAC tumours are characterised by a voluminous stroma that is rich in extracellular matrix (ECM) molecules such as collagens, hyaluronan (HA) and matricellular proteins. These stromal components have been suggested to promote PDAC cell migration, proliferation, evasion of apoptosis and chemotherapy resistance. Those events are mediated via interactions with adhesion receptors, such as integrins and CD44 receptors expressed on cancer cell surfaces.

Micro-RNAs (miRNA) post-transcriptionally regulate gene expression in health and disease. At the time of PDAC diagnosis, miRNA levels are altered both in plasma and tumour tissue. Before PDAC diagnosis, tissue miRNA levels are altered in precursor lesions, raising the possibility that plasma miRNAs might aid in early detection.

In this thesis, it is hypothesised that stromal components and miRNAs can serve as tissue or blood based biomarkers in PDAC. The aims are: (1) to characterise the expression of stromal components and their receptors in normal and cancerous tissue; (2) to find potential stroma-associated tissue and blood-based biomarkers for diagnosis and prognosis estimates; (3) to determine the cellular effects of type IV collagen (Col IV) in PDAC; (4) to determine if plasma miRNAs that are altered in manifest PDAC can be used to diagnose PDAC earlier.

Methods
The expression patterns of Col IV, Col IV-binding integrin subunits (α1, α2, β1), Endostatin, Osteopontin (OPN) and Tenascin C (TNC) were analysed in frozen PDAC and normal pancreatic tissue. A tissue microarray (TMA) was constructed using formalin-fixed, paraffin-embedded primary tumours and lymph node metastases. The TMA was used to study the expression levels and associations with survival of the standard CD44 receptor (CD44s), its variant isoform 6 (CD44v6), HA, OPN and Col IV. Circulating levels of HA, Col IV, Endostatin, OPN and TNC were measured in PDAC patients and healthy individuals, and compared with conventional tumour markers (Ca 19-9, CEA, Ca 125 and TPS). The functional roles of Col IV were studied in PDAC cell lines by: (1) growth on different matrices (2) blocking Col IV
binding integrin subunits, (3) blocking the Col IV domains 7s, CB3 and NC1, and (4) by down regulation of PDAC cell synthesis of Col IV using siRNA transfection. Plasma miRNAs alterations were screened for in samples from patients with manifest disease, using real-time quantitative PCR (RT-qPCR). To find early miRNA alterations, levels of those miRNAs that were altered at diagnosis were measured in prediagnostic plasma samples.

**Results**

High tissue expression of both the standard CD44 receptor (CD44s) and its variant isoform CD44v6 as well as low expression of stromal OPN were associated with poor survival. In addition, high CD44s and low OPN predicted poor survival independent of established prognostic factors.

Circulating Col IV, Endostatin, OPN, TNC and HA were increased in preoperative samples from PDAC patients. Preoperatively, higher levels of serum-HA and plasma-Endostatin were associated with shorter survival. Postoperatively, higher levels of Col IV, Endostatin and OPN were associated with shorter survival. On the contrary, only one of the conventional tumour markers was associated with survival (Ca 125).

Col IV stimulated PDAC cell proliferation and migration and inhibited apoptosis in vitro, dependent on the collagenous domain (CB3) of Col IV and the Col IV binding integrin subunit β1. Reduced endogenous Col IV synthesis inhibited these effects, suggesting that PDAC cells synthesise Col IV to stimulate tumour-promoting events via a newly discovered autocrine loop.

15 miRNAs were altered in early stage PDAC patients and the combination of these markers outperformed Ca 19-9 in discriminating patients from healthy individuals. However, none of the miRNAs were altered in prediagnostic samples, suggesting that plasma miRNA alterations appear late in the disease course.

**Conclusions**

Up regulated stromal components in PDAC tumours are detectable in blood samples and are potential diagnostic and prognostic biomarkers in PDAC. High circulating levels of Col IV, Endostatin, OPN and HA predict poor survival, as well as high expression of CD44s and CD44v6 and low expression of OPN in tumour tissue. PDAC cells synthesise Col IV, which forms BM-like structures close to cancer cells and promote tumour progression in vitro via an autocrine loop. Several plasma-miRNAs are altered in PDAC, but are not useful for early discovery.
List of abbreviations

5-FU  5-fluorouracil
7s The N-terminal domain on collagen molecules
α-SMA alpha smooth muscle actin
AUC Area under the curve
BM Basement membrane
Ca 19-9 Carbohydrate antigen 19-9
Ca 125 Cancer antigen 125
CB3-domain The collagenous domain on collagenous molecules
CD44 Cluster of differentiation 44
CD44v6 CD44 variant isoform 6
CEA Carcinoembryonic antigen
CK 18 Cytokeratin 18
Col IV Type IV collagen
Col XVIII Type XVIII collagen
ECM Extracellular matrix
ELISA Enzyme-linked immunosorbent assay
EMT Epithelial to mesenchymal transition
FFPE Formalin-fixed and paraffin-embedded
FGF Fibroblast growth factor
GEM Gemcitabine
HA Hyaluronan
HABP Hyaluronic acid binding protein
hENT1 Human equilibrative nucleoside transporter 1
HGF Hepatocyte growth factor
IF Immunofluorescence
IHC Immunohistochemistry
In vitro Cell studies outside their biological context
In vivo Experiments on living organisms
IPMN Intraductal papillary mucinous neoplasm
KRAS Kirsten rat viral sarcoma oncogene
M Molecular mass
MAPK Mitogen-activated protein kinase
MCN Mucinous cystic neoplasia
mRNA Messenger RNA
miRNA Micro-RNA
MMP Matrix metalloproteinase
MRI Magnetic resonance imaging
NC1 The non-collagenous domain on collagen molecules
OPN Osteopontin
PanIN Pancreatic intraepithelial neoplasia
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>PDAC</td>
<td>Pancreatic ductal adenocarcinoma</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PLA</td>
<td>Proximity ligation assay</td>
</tr>
<tr>
<td>PSC</td>
<td>Pancreatic stellate cells</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver operating characteristics</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RT qPCR</td>
<td>Real-time quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SHH</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue microarray</td>
</tr>
<tr>
<td>TNC</td>
<td>Tenascin C</td>
</tr>
<tr>
<td>TPS</td>
<td>Tissue polypeptide specific antigen</td>
</tr>
<tr>
<td>VIP</td>
<td>The Västerbotten intervention program</td>
</tr>
</tbody>
</table>
List of original papers

**Paper I**

**Paper II**

**Paper III**
Franklin O, Billing O, Öhlund D, Berglund A, Wang W, Hellman U, Sund M. CD44 receptors and stromal CD44 ligands as prognostic markers in pancreatic ductal adenocarcinoma. (Manuscript)

**Paper IV**
INTRODUCTION

The focus of this thesis is the tumour stroma and micro-RNAs as sources for biomarker discovery in pancreatic cancer, as well as the pathophysiological role of the stromal basement membrane protein type IV collagen.

The first two chapters in the introduction will provide a brief overview of the normal pancreas and pancreatic cancer. This is followed by an introduction of the concept of tumour biomarkers in pancreatic cancer in chapter three. In chapter four the tumour stroma in pancreatic cancer is introduced, with emphasis on its pathophysiological role. In chapter five, stromal components and cell receptors that are studied in this thesis are introduced. Finally, chapter six will introduce micro-RNAs and implications for their use as biomarkers for early detection of pancreatic cancer.

Chapter 1 - The pancreas

1.1 Anatomy of the normal pancreas

The pancreas is a retroperitoneal organ that extend horizontally along the posterior abdominal wall at lumbar spine level L1-2. It is roughly J-shaped and can be divided into the head (caput), body (corpus) and tail (cauda). The main pancreatic duct (ductus pancreaticus) runs from head to tail and fuses with the common bile duct in the pancreas head. The fused ducts enter the duodenum via the Papilla of Vater to excrete pancreatic juice and bile into the gastrointestinal canal. The pancreas lies proximal to large arteries and veins that provides blood supply to the organ and are of importance in pancreatic cancer staging and surgery. These include branches from the celiac axis (truncus coeliacus), the splenic artery, the superior mesenteric vein and artery and the portal vein (Drake et al, 2015) (Figure 1A).

1.2 The functional components of the normal pancreas

The pancreas has two glandular components – the endocrine and the exocrine pancreas (Figure 1B). The endocrine pancreas resides in islets of Langerhans, cell clusters that synthesise hormones involved in carbohydrate, fat and protein metabolism, including insulin and glucagon (Ross & Pawlina 2006). The exocrine pancreas constitutes >90% of the organ and secrete enzymes involved in food digestion. This thesis focuses on pancreatic ductal adenocarcinoma, that originate from epithelial cells in the exocrine pancreas.
1.2.1 The exocrine pancreas

The exocrine pancreas is made up of secretory glands, acini, composed of acinar cells, and ductal systems that transport acinar cell secretions. The acinar cells secrete zymogen granules that are transported in the pancreatic juice via ducts lined by ductal epithelium, to end up in the gastrointestinal canal via the main pancreatic duct (Figure 1C). The zymogen granules contain enzymes that serve to digest proteins, carbohydrates, nucleic acids and lipids in the food. The protein digesting enzymes (endopeptidases) are inactive until they reach the duodenal mucosa to prevent auto digestion of the organ (Ross & Pawlina 2006). Obstructing gall stones, excessive alcohol

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**Figure 1. Anatomy and histology of the pancreas.** A) The anatomy of the pancreas and adjacent anatomical landmarks. B) Schematic illustration of the pancreas histology. C) Close-up of an individual acini surrounded by PSCs. Illustration by G. Andersson. Adapted from Öhlund (2010). ©Umeå University. Used with permission.
intake and tissue trauma can cause disturbances in this system leading to auto-digestion and acute pancreatitis. Longstanding tissue stress and inflammation cause chronic pancreatitis, characterised by organ fibrosis (Banks et al, 2010).

Pancreatic stellate cells (PSCs) are located in the basal aspect of acinar cells and are characterised by cytoplasmic lipid depositions and long cytoplasmic projections that extend along adjacent acini (Figure 1C). In the normal pancreas, PSCs are quiescent and few in number. Pancreatic injury or tissue stress activates PSCs, which result in morphological changes, increased proliferation and increased synthesis of extracellular matrix components. This is central to the development of a distorted fibrosis (desmoplasia), that is typical for the stroma in both chronic pancreatitis and pancreatic cancer (Erkan et al, 2012). The PSCs will be further discussed in the context of the tumour stroma in chapter 4.

Chapter 2 - Pancreatic Cancer

Of the different tumour types that arise in the pancreas, the most common is pancreatic ductal adenocarcinoma (Kamisawa et al, 2016), and the focus of this thesis. Herein, the terms pancreatic cancer and pancreatic ductal adenocarcinoma (PDAC) are used synonymously.

2.1 Epidemiology

In Sweden, 1251 patients were diagnosed with pancreatic cancer in 2014, with a peak incidence in the ages between 60-80 years (Socialstyrelsen 2015). In the United States, it is the 12th most common cancer form, but despite its relatively low incidence it is the fourth most common cause of cancer related deaths (Siegel et al, 2016). Risk factors include smoking, chronic pancreatitis, diabetes mellitus, obesity as well as up to a 13-fold increased risk in individuals with certain genetic syndromes such as Peutz-Jeghers syndrome and BRCA2 syndrome (Wolfgang et al, 2013). However, most PDAC tumours are not caused by inherited mutations, but occur sporadically due to extrinsic factors, such as carcinogens, or intrinsic factors, such as random mistakes during DNA replication (Vincent et al, 2011; Makohon-Moore & Iacobuzio-Donahue 2016).

2.2 Survival

Pancreatic cancer patients have the worst long term survival rates among all cancer forms (Table 1) (Siegel et al, 2016). The main reason is that most patients are diagnosed with metastatic or locally advanced disease, when curative surgery is unfeasible (Kamisawa et al, 2016). As a consequence, only 2 out of 10 patients are diagnosed with early stage disease and can be offered surgery with curative intent. However, early tumour relapse is common after

3
surgery. The median postoperative survival for early staged patients that undergo surgery is less than 2 years, the five-year survival is 27% and only one out of eight are actually cured (Bilimoria et al, 2007; Schnelldorfer et al, 2008; Siegel et al, 2016). Moreover, the prognosis has barely improved at all during the past 30 years, while a substantially improved survival has been accomplished in other cancer forms, such as breast and prostate cancer (Siegel et al, 2016). Consequently, pancreatic cancer is expected to become the second most common cause of cancer related deaths by the year 2020 (Rahib et al, 2014).

The survival statistics clearly shows that the current diagnostics, staging system and treatments are insufficient in providing PDAC patients with decent medical care. Improving the survival requires; strategies for early detection, increased knowledge about the underlying tumour biology, determination of better prognostic factors and more effective treatments.

Table 1. The 5-year survival of the four most common cancer forms compared to pancreatic cancer, by disease stage (Siegel et al., 2016).

<table>
<thead>
<tr>
<th>Stage</th>
<th>PDAC</th>
<th>Lung</th>
<th>Breast</th>
<th>Prostate</th>
<th>Colorectal</th>
</tr>
</thead>
<tbody>
<tr>
<td>All stages</td>
<td>8 %</td>
<td>18 %</td>
<td>91 %</td>
<td>99 %</td>
<td>66 %</td>
</tr>
<tr>
<td>Localised</td>
<td>27 %</td>
<td>55 %</td>
<td>99 %</td>
<td>&gt;99 %</td>
<td>90 %</td>
</tr>
<tr>
<td>Regional</td>
<td>11 %</td>
<td>27 %</td>
<td>85 %</td>
<td>&gt;99 %</td>
<td>71 %</td>
</tr>
<tr>
<td>Metastatic</td>
<td>2 %</td>
<td>4 %</td>
<td>26 %</td>
<td>28 %</td>
<td>13 %</td>
</tr>
</tbody>
</table>

2.3 Diagnosis and tumour staging
PDAC is generally asymptomatic until it impacts on adjacent tissue or metastasise. Common symptoms of PDAC include fatigue (86%), weight loss (85%), abdominal pain (79%), dark urine (59%) and jaundice (56%). Jaundice is a common clinical sign in patients with early stage disease and presents as yellowing skin and episclera. This is due to hyperbilirubinemia secondary to tumour obstruction of the common bile duct (Porta et al, 2005).

Imaging modalities for PDAC detection include contrast-enhanced computed tomography (CT), magnetic resonance imaging (MRI), magnetic resonance cholangiopancreatography (MRCP), and ultrasound (abdominal or endoscopic) (Lee & Lee 2014). The main determinant of resectability is involvement of nearby arteries and veins, as long as no metastases are discovered. The disease is staged according to the TNM staging system (Table 2). Stage I-II patients are candidates for surgery, whereas stage III patients are considered borderline resectable. Stage IV equals metastatic cancer and is not curable with surgery (Appel et al, 2012).
Table 2. TNM staging for pancreatic cancer

<table>
<thead>
<tr>
<th>TNM stage</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA</td>
<td>Tumour limited to the pancreas, size ( \leq 2 \text{ cm} ) (T1)</td>
</tr>
<tr>
<td>IB</td>
<td>Tumour limited to the pancreas, size ( &gt; 2 \text{ cm} ) (T1)</td>
</tr>
<tr>
<td>IIA</td>
<td>Tumour extends beyond the pancreas but does not involve SMA or the celiac axis (T3)</td>
</tr>
<tr>
<td>IIB</td>
<td>Regional lymph node metastasis (N1)</td>
</tr>
<tr>
<td>III</td>
<td>Tumour involves the celiac axis or SMA (T4)</td>
</tr>
<tr>
<td>IV</td>
<td>Distant metastasis (M1)</td>
</tr>
</tbody>
</table>

SMA = superior mesenteric artery. Modified from Appel et al., 2012

2.4 Surgical treatment

Surgery is commonly performed as a pancreaticoduodenectomy (Whipple’s procedure) for tumours in the head, or a distal pancreatectomy for tail tumours. Here follows a brief description of the Whipple procedure.

First the abdominal cavity is entered with a midline incision and examined to exclude eventual metastasis missed by CT/MRI. Then the large vessels are exposed followed by resection of the pancreatic head and regional lymph nodes, the entire duodenum, a distal portion of the stomach, the proximal jejunum, the common bile duct and the gall bladder. The specimen is removed \textit{en bloc}. Finally, gastrointestinal continuity is restored by connecting the common hepatic duct, the remaining pancreas and the remaining stomach with jejunum (Figure 2).

![Figure 2. Pancreaticoduodenectomy (Whipple’s procedure).](image)

The anatomical locations for resection (A) and reconstruction (B). Adapted from Wolfgang et al, 2013. Illustration by Corinne Sandone. © Johns Hopkins University; used with permission.
It takes roughly 2-3 months to recover from the operation and the perioperative mortality rate is 2-3 % (Wolfgang et al, 2013). Evidence for the benefit of surgery is purely observational; an improved survival has been shown in resected patients vs. non-resected at early stages and there are reports of 100% 5-year survival in <1 cm sized tumours (Ariyama et al, 1998; Wagner et al, 2004; Bilimoria et al, 2007). In order to survive pancreatic cancer, an early diagnosis and radical surgery is crucial.

2.5 Oncological treatment

Oncological (non-surgical) treatments can be divided into treatment with curative or palliative intent. Curative treatment is given as a complement to surgery for patients with localised disease and is either given before (neoadjuvant therapy) or after surgery (adjuvant therapy). Palliative treatment is offered patients with advanced or metastatic disease that are not eligible for surgery.

2.5.1 Chemotherapy with curative intent

Adjuvant chemotherapy is offered to all patients that recover from surgery. The standard regimens are gemcitabine (GEM) or fluorouracil based chemotherapy (5-FU), which are equally effective (Neoptolemos et al, 2010). There are evidence for 5-year survival benefits with these therapies, compared to placebo (21-29% vs. 10-11%), but the median survival difference is only a few months (22-23 vs. 17-20 months) (Neoptolemos et al, 2004; Oettle et al, 2013). This indicate that only a subset of patients truly benefits from these treatments. While a plethora or studies have failed to find better options, a recently published Japanese trial showed remarkable results for S-1, an oral fluorouracil pro-drug compared to GEM. The study was discontinued at interim analysis since the S-1 arm had a remarkably higher median survival (46.5 months vs. 25.5) and 5-year survival rates (44% vs. 24%) (Uesaka et al, 2016). Oral S-1 therapy is currently being evaluated in western populations since the pharmacodynamics and kinetics differ.

Neo-adjuvant therapy for PDAC is a controversial subject. In favour of preoperative chemotherapy is a higher chance of a radical resection, but it also delays potentially curative surgery. There is currently no strong evidence advocating neo-adjuvant therapy for resectable nor borderline resectable PDAC (Kamisawa et al, 2016).

2.5.1 Palliative chemotherapy

The most effective palliative treatment for metastatic PDAC patients is the combination of 5-FU, leucovorin, irinotecan and oxaliplatin (FOLFIRINOX), demonstrated to be superior to GEM (median survival 11.1 vs. 6.8 months) in the ACCORD-11 trial (Conroy et al, 2011). However, the external validity of that study is limited by its strict inclusion criteria (patients aged ≤ 75 years,
good performance status, no cardiac disease an low risk of developing cholestasis). The MPACT study showed a less impressive median survival improvement with albumin bound (nab)-paclitaxel plus GEM (8.5 vs 6.7 months vs. GEM alone) but had less strict inclusion criteria (Von Hoff et al, 2013). Additionally, nab-paclitaxel+GEM was associated with less severe adverse advents than FOLFIRINOX, advocating its use in patients unfit for FOLFIRINOX treatment. Studies evaluating the use of FOLFIRINOX and Nab-paclitaxel in the adjuvant setting are ongoing.

2.6 The PDAC histopathology
Pancreatic ductal adenocarcinoma is believed to develop from ductal cells in the normal pancreas, although in vitro and in vivo studies have suggested that it might develop from acinar cells undergoing ductal metaplasia (Rooman & Real 2012). Under the microscope, the malignant cells form duct-like glandular structures of varying differentiation grades, surrounded by stromal desmoplasia; a voluminous tumour stroma consisting of distorted fibrosis (figure 3B) (described further in chapter 4). Other characteristics include a haphazard growth pattern violating the tissue architecture, vessel and nerve invasion, nuclear pleomorphism, necrotic debris within the glands and disrupted glandular lumina. The tumours are graded as well-differentiated (Grade 1), moderately differentiated (Grade 2) and poorly differentiated (Grade 3) based on the glandular architecture, mitosis frequency and nuclear pleomorphism (Hruban & Fukushima 2007).

2.6.1 Precursor lesions
PDAC is preceded by precursor lesions called pancreatic intraepithelial neoplasias (PanIN). PanINs progress from low-grade dysplasia (PanIN-1 to PanIN-2) into high-grade dysplasia (PanIN-3). PanIN-3 resemble PDAC but lack signs of invasion (Haugk 2010). Unfortunately, current imaging
modalities cannot effectively discover PanIN-lesions (Lee & Lee 2014). On the contrary, radiology can discover cystic tumours that occasionally progress to pancreatic cancer, such as intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs) (Haugk 2010).

IPMN is the most common entity of the two tumours and ~10-30% of IPMNs progress into invasive carcinoma (Hackert et al, 2015). The current European guidelines suggest surgical resection of MCNs, IPMNs involving the main duct, and IPMNs in ductal branches if certain risk factors are present, to prevent progression into lethal pancreatic cancer (Del Chiaro et al, 2013).

**2.7 Pathogenesis and malignant progression**

The progression from normal epithelial cells via precursor lesions to pancreatic cancer cells with metastatic potential involves the acquisition of certain capabilities – hallmarks of cancer. These include sustained proliferative signalling, evasion of growth suppression, resisting apoptosis, inducing angiogenesis, enabling replicative immortality and activating invasion and metastasis. The hallmarks arise from mutations leading to up regulation of oncogenes and down regulation of tumour suppressors, thereby governing the tumour behaviour (Hanahan & Weinberg 2011).

The four main driver mutations in PDAC are the tumour suppressor genes TP53, SMAD4/DPC4 and CDKN2A/p16 and the oncogene KRAS. KRAS is mutated in >90% of PDAC tumours. Additionally, a large number of more infrequent mutations have been described, highlighting the heterogeneous mutational landscape in PDAC (Kanda et al, 2012; Makohon-Moore & Iacobuzio-Donahue 2016).

**KRAS** mutation is an early mutational event, evident by a high prevalence already in PanIN1-lesions. However, a **KRAS** mutation alone is insufficient for PDAC progression, and additional driver mutations follow during PanIN-progression and clonal expansion, such as loss of CDK2NA/p16 in PanIN-2 and loss of TP53, SMAD4/DPC4 and BRCA2 in PanIN-3 lesions (Maitra et al, 2003) (Figure 4). The PanIN to PDAC progression has been recapitulated in transgenic mice with inducible **KRAS** and **TP53** mutations (KPC mice) (Hingorani et al, 2005). Interestingly, the progression is paralleled with altered expression of several micro-RNAs (LaConti et al, 2011; Yu et al, 2012), described in more detail in chapter 6.

PDAC tumours most commonly metastasise to the peritoneal cavity, lungs and the liver (Wolfgang et al, 2013). Metastasising cancer cells undergo phenotypical changes called epithelial to mesenchymal transition (EMT). EMT is characterised by an altered expression of adhesion molecules, a mesenchymal-like morphology and an up regulation of transcription factors that are involved in this process (Lamouille et al, 2014). Interestingly, PanIN
cells can undergo EMT and seed to distant organs even before PDAC develops in mouse models, indicating that the metastasis cascade might occur early in the PDAC progression (Rhim et al, 2012).

![Figure 4. The progression from normal epithelia to PDAC via precursor lesions. Examples of common mutational events are indicated. Adapted from Maitra et al., 2003. © Macmillan Publishers Limited, used with permission.]

Others claim that metastasis occur late in the progression. Yachida et al. calculated time intervals in PDAC progression based on the differences and similarities in the mutational profiles of autopsied primary tumours and metastases. They concluded that distant metastasis is a late event in pancreatic cancer, with over a decade from the first driver mutation to metastasis initiation (Yachida 2010). This suggests that there is a window of opportunity for early detection of PDAC.

Chapter 3 – Biomarkers in pancreatic cancer

In this thesis, potential prognostic and diagnostic PDAC biomarkers for are sought in tissue and in the circulation. In this chapter the concept of biomarkers is introduced. The strengths and weaknesses of the clinically implemented PDAC biomarker carbohydrate antigen 19-9 (Ca 19-9) are presented, as well as other clinically used biomarkers that are included in paper 2.

3.1 The concept of biomarkers

Biomarkers can potentially aid in early detection, in differential diagnostics, by predicting treatment response and by monitoring disease relapse. A definition has been proposed by the World Health Organisation as: “any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease” (WHO 2001).
The clinical uses of biomarkers can broadly be divided into:

1. **Screening markers** – used on a population basis, aiming to diagnose the condition early, either among asymptomatic individuals or in selected risk populations
2. **Diagnostic markers** – used when a disease is suspected, aiming to differentiate between resembling conditions in symptomatic individuals
3. **Prognostic markers** – used after a clinical diagnosis or strong suspicion, aiming to stage the disease properly with regards to outcome, such as recurrence or survival
4. **Predictive markers** – used before treatment, aiming to predict the treatment that the patient is most likely to benefit from
5. **Monitoring markers** – used after treatment, aiming to detect disease recurrence

The accuracy, measured as the sensitivity and specificity, is crucial in all aspects of biomarker use. The sensitivity measures the ability of a test to correctly classify diseased individuals as having the disease (true positive rate). The specificity measures the ability of a test to correctly classify healthy individuals as not having the disease (true negative rate).

### 3.2 PDAC biomarkers

Numerous potential diagnostic and predictive blood based biomarkers for PDAC have been reported in literature, but none have reached widespread clinical use except Ca 19-9. Moreover, no tissue biomarker is routinely used to aid in clinical decision making regarding treatment strategies. The most promising tissue biomarker is the human equilibrative nucleoside transporter 1 (hENT-1). Patients with low hENT-1 expressing tumours was convincingly shown to respond poorly to adjuvant GEM (Greenhalf et al, 2014), although prospective validation is required before clinical implementation. Moreover, an increasing number of publications have reported on the potential use of miRNAs as both tissue and blood-based biomarkers in PDAC (introduced in chapter 6).

#### 3.2.1 Carbohydrate antigen 19-9 (Ca 19-9)

Circulating Ca 19-9 is the most thoroughly studied and clinically used PDAC biomarker, and is currently the “golden standard”, that new biomarkers are judged against. Ca 19-9 is a glycolipid that is expressed on pancreatic cancer cell surfaces, and its normal counterpart - diasyl Lewis-a, plays a role in immune surveillance on the epithelial surface of various gastrointestinal organs. Aberrant Ca 19-9 synthesis stems from epigenetic silencing of the
sialyl transferase enzyme during early cancer progression, leading to an abnormal tissue accumulation and increase of Ca 19-9 in the circulation. High Ca 19-9 levels is strongly associated with advanced tumour stage, non-resectability, poor treatment response and poor overall survival in PDAC. But despite a strong association with outcome, the clinical usefulness of Ca 19-9 is limited (Ballehaninna & Chamberlain 2012).

As a diagnostic marker Ca 19-9 reaches a sensitivity and specificity of 78-81% and 83-90% respectively, for discriminating between PDAC and healthy patients or patients with benign pancreatic disease (Steinberg 1990; Poruk et al, 2013). Ca 19-9 synthesis occurs only in individuals that belong to the Lewis blood group. Hence, 5-10% of PDAC patients cannot express the Ca 19-9 antigen, which contribute to the false negative rate (Ballehaninna & Chamberlain 2012). False positive elevations have been reported in various malignant and non-malignant conditions including colorectal cancer, gastric cancer, pancreatitis, liver cirrhosis and rheumatoid arthritis, which limits the specificity of Ca 19-9. Additionally, Ca 19-9 increases in hyperbilirubinemia secondary to both benign and malignant causes of bile duct obstruction, adding yet a confounder to the interpretation (Ballehaninna & Chamberlain 2012).

Two studies have prospectively evaluated and concluded that Ca 19-9 is ineffective as a screening marker in asymptomatic populations (Kim et al, 2004; Chang et al, 2006). The positive predictive values were 0,5 % and 0,9 % respectively in these studies, which means that less than 1% with increased Ca 19-9 in a population screening would truly have PDAC. Additionally, two studies have shown that Ca 19-9 increases within 1-2 years prior to diagnosis, by analysing samples collected from PDAC patients before diagnosis. In both studies, the sensitivity and specificity of Ca 19-9 was low prior to diagnosis (Nolen et al, 2014; O’Brien et al, 2015).

While being somewhat useful in disease monitoring and as a complement at the diagnostic work-up, the use of Ca 19-9 alone as a biomarker in PDAC management is limited.

3.2.2 Tissue polypeptide specific antigen (TPS)
TPS is a fragment of cytokeratin 18 (CK18), which is an integral component of the epithelial cytoskeleton of both normal and malignant cells. TPS is released into the circulation upon cell proliferation and apoptosis and has been suggested to reflect tumour growth. In breast, ovarian, prostate and lung cancer, TPS levels can predict outcome and response to therapy (Barak et al, 2004). In PDAC, TPS has been shown to better differentiate between PDAC and chronic pancreatitis than Ca 19-9, reaching high sensitivity and specificity (98%/97%) for discriminating between the conditions (Slesak et al, 2000).
3.2.3 Carcinoembryonic antigen (CEA)
CEA is a glycoprotein involved in cell adhesion that is widely expressed in the gastrointestinal mucosa. Despite a low accuracy, circulating CEA is widely implemented in the clinical management of colorectal cancer (Sorensen et al, 2016). In PDAC, a systematic review has concluded that CEA has slightly higher specificity than Ca 19-9 in differentiating between PDAC and non-malignant pancreatic diseases, but at the cost of a lower sensitivity (Poruk et al, 2013).

3.2.4 Cancer antigen 125 (Ca 125)
Ca 125 is an epitope on the mucin 16 protein (MUC16), which is expressed on mucosal epithelial membranes. Ca 125 is used clinically in the diagnosis and disease monitoring of ovarian cancer (Felder et al, 2014). In PDAC, Ca 125 is elevated compared to benign pancreatic tumours (Cwik et al, 2006) and has been suggested to be a more reliable prognostic marker than Ca 19-9 (Luo et al, 2013).

3.3 Biomarker combinations
Since Ca 19-9 has obvious downsides as a PDAC biomarker, studies have looked into biomarker combinations to improve the diagnostic and prognostic value compared to Ca 19-9 alone. Two studies recently reported that the combination of Ca 19-9 and CEA predict survival better than Ca 19-9 alone (Kanda et al, 2014; Reitz et al, 2015). Chan et al. showed that the combination of Ca 125, Ca 19-9 and the BM protein laminin subunit gamma 2 outperformed Ca 19-9 alone at detecting early stage PDAC (Chan et al, 2014). Another recent study suggested that patients with high Ca 19-9 levels (≥1000 U/mL) have a questionable benefit of surgery. As a group, those patients did not live longer than non-resected patients with advanced PDAC. However, patients where high Ca 19-9 decreased postoperatively did benefit from surgery. The authors showed that Ca 125 combined with CEA could predict a postoperative Ca 19-9 decrease with high accuracy, thereby determining the indication for surgery (Liu et al, 2015). These studies highlight that combining several circulating biomarkers can provide additional information compared to a single marker.

Chapter 4 - The tumour stroma in pancreatic cancer

In PDAC, up to 80% of the tumour volume is composed of tumour stroma (Figure 3). The stroma refers to the non-epithelial cells (fibroblasts, immune cells, nerve cells, endothelial cells) and the fibrous ECM components they collectively produce. This pathological expansion of fibrous ECM is called stromal desmoplasia (Moir et al, 2015). The desmoplastic
PDAC stroma contains numerous structural and functional molecules that directly affect cancer cell functions; including collagens, hyaluronan and matricellular proteins (introduced in chapter 5). This chapter introduces the etiology and the pathophysiological role of the tumour stroma in PDAC.

4.1 Activated pancreatic stellate cells
The main source of the ECM production and desmoplasia in PDAC are activated pancreatic stellate cells (PSCs) (Apte et al, 2004). Upon activation PSCs acquire a myofibroblast-like phenotype associated with an increased ECM production, secretion of tumour promoting growth factors and expression of α-smooth muscle actin (αSMA) (Erkan et al, 2012; Sherman et al, 2014). Human PDAC tumours display a high number of αSMA-positive cells in the stroma, indicative of a widespread PSC activation and proliferation (Bachem et al, 2005). High αSMA expression in PDAC tumours have been associated with shorter survival in some reports (Fujita et al, 2010; Sinn et al, 2014), while other studies could not find significant associations between αSMA and prognosis (Erkan et al, 2008; Bever et al, 2015; Wang et al, 2016).

4.2 Cancer cell – stroma interactions
PDAC cells and PSCs has been shown to stimulate each other in a paracrine manner in vitro to cooperate in PDAC carcinogenesis. Central in this interplay is the protein sonic hedgehog (SHH). While absent in the normal pancreas, SHH is overexpressed in PanIN lesions and PDAC cells (Thayer et al, 2003), and has been shown to activate quiescent PSCs and induce desmoplasia via the hedgehog pathway (Bailey et al, 2008; Tape et al, 2016). PSC proliferation and ECM synthesis is further stimulated by growth factors released from PDAC cells, such as platelet derived growth factor (PDGF), Transforming Growth factor beta-1 (TGFβ1) and Fibroblast Growth factor (FGF) (Apte et al, 1999; Lohr et al, 2001; Bachem et al, 2005). In turn, activated PSCs reciprocally induce gene expression and protein expression changes in PDAC cells that stimulate their proliferation, migration and evasion of apoptosis. These effects are mediated by secreted growth factors, such as Hepatocyte Growth Factor (HGF) and PDGF from activated PSCs (Hwang et al, 2008; Vonlaufen et al, 2008; Kadaba et al, 2013; Pothula et al, 2015), and stromal components such as collagens, hyaluronan and matricellular proteins (Figure 5).

Mouse models have added in vivo evidence of the malignant interplay between PDAC cells and PSCs. Injecting PDAC cells together with PSCs into nude mice increases desmoplasia, tumour growth and the rate of metastatic foci at distant organs compared to injection of PDAC cells alone (Bachem et al, 2005; Hwang et al, 2008; Vonlaufen et al, 2008; Pothula et al, 2016).
Additionally, PSCs co-migrate with PDAC cells to metastatic sites where they induce a desmoplastic stroma, rich in stromal components that have been associated with poor survival (Xu et al., 2010; Whatcott et al., 2015). The desmoplastic stroma reduces vessel density in human PDAC tissue, but also in transgenic mouse models, where it contributes to reduced chemotherapy deliverance (Olive et al., 2009; Chauhan et al., 2013). Hedgehog signalling inhibition as well as depletion of stromal hyaluronan reduce desmoplasia, increase GEM deliverance and prolong survival in PDAC mouse models (Olive et al., 2009; Jacobetz et al., 2012; Provenzano et al., 2012; Chauhan et al., 2013).

**Figure 5. The malignant interplay between the cancer cells and activated PSCs in PDAC tumours.** PSCs are activated and stimulated by SHH and growth factors released from PDAC cells. This leads to PSC proliferation and increased synthesis of growth factors and stromal ECM components that in turn stimulate PDAC cell proliferation, survival, migration and chemoresistance.

Most published studies support the hypothesis that activated PSCs and stromal components in the microenvironment stimulate cancer cells and contribute to the progression, invasion and chemotherapy resistance in PDAC. Hence, tumour stroma targeting is an attractive treatment strategy (Bijlsma & van Laarhoven 2015).

But in the recent years this dogma has been challenged in three aspects. While there is general agreement that PSCs promote stromal desmoplasia, conflicting evidence regarding the pathophysiological role of the stroma have recently surfaced. First, human PDAC tumours with a dense stroma and high collagen content has been associated with improved survival in some studies (Erkan et al., 2008; Bever et al., 2015; Wang et al., 2016). Second, clinical
trials aiming to target the tumour stroma in patients have not yet proven any benefit, and a hedgehog inhibition phase II trial was stopped because of worse outcome in the treatment arm (Bijlsma & van Laarhoven 2015). Third, three papers published in 2014 used different strategies to inhibit the formation of the desmoplastic stroma in transgenic mouse models (deleting the SHH gene, hedgehog signalling inhibition, or depletion of the αSMA+ cell population). In all three studies, this led to more aggressive tumours and reduced animal survival (Lee et al, 2014; Ozdemir et al, 2014; Rhim et al, 2014). Collectively, this has spurred a paradigm shift. The stroma is now considered to contain both tumour promoting and suppressive components.

Chapter 5 – The stroma as a source of biomarkers

The desmoplastic stroma is an ubiquitous feature of PDAC tumours and stromal components have functional roles in carcinogenesis. Hence, the PDAC stroma could potentially harbour clinically useful biomarkers of disease, complementing cancer cell derived markers. This chapter aim to introduce certain stromal molecules and receptors of relevance within this thesis.

5.1 Hyaluronan (HA)

Hyaluronan (HA) is a glycosaminoglycan family member, composed of repeating disaccharides (N-acetylglucosamine and glucuronic acid) and is abundant in the ECM of both healthy and malignant tissues. HA is synthesised at the cell membrane by hyaluronic acid synthases (HAS 1-3) and is degraded by hyaluronidases (HYAL 1-2). The HA regulation machinery is poorly understood, but the balance between HA synthesis and breakdown leads to HA of varying molecular mass. High molecular mass (M) HA has a high capacity for binding water molecules. This space occupying property is important in many tissue, such as the skin, the vitreous body of the eye and joints. Low M HA is more frequent in inflamed tissues and these fragments have been suggested to be angiogenic and tumour promoting (Toole 2004; Stern et al, 2006).

5.1.1 Hyaluronan in cancer

In cancer, HA is involved in cell survival, metastasis, angiogenesis and multidrug resistance. Such functions depend on its tissue occupying properties but also on interactions with cellular receptors such as CD44 and RHAMM (Toole 2004; Sironen et al, 2011). HA-CD44 signalling pathways in cancer will be discussed in a later section of this chapter, dedicated to the CD44 receptor in cancer cell – stroma interactions (5.2.1).
5.1.2 Hyaluronan in pancreatic cancer

PDAC tumours are particularly rich in HA when compared to other solid tumours (Theocharis et al, 2000; Jacobetz et al, 2012) and both high M HA and low M HA increase cell migration in vitro (Cheng et al, 2016). Additionally, its mechanical properties play an important role in vivo. A high stromal deposition of HA increases the interstitial fluid pressure within the tumour, leading to vascular collapse and impaired perfusion of chemotherapeutic agents. Stromal HA can be depleted in mouse models by administering hyaluronidases or hypertensive medication (angiotensin-II receptor blockers). This reduce tumour growth, increase chemotherapy penetrance and prolong animal survival (Jacobetz et al, 2012; Provenzano et al, 2012; Chauhan et al, 2013). Moreover, hyaluronidase treatment is well tolerated by patients with stage IV PDAC, demonstrated in a recent phase I trial (Hingorani et al, 2016). Two ongoing clinical phase II studies will evaluate the potential survival benefits of hyaluronidase treatment in combination with FOLFIRINOX or nab-paclitaxel in stage IV PDAC (clinicaltrials.gov, study IDs: NCT01839487 and NCT01959139).

High tissue expression of stromal HA has been associated with poor prognosis in several cancer forms (Tammi et al, 2008), including PDAC (Cheng et al, 2013; Whatcott et al, 2015). However, those two studies had major methodological flaws. The study by Cheng et al. used a commercial HA antibody to detect HA (Cheng et al, 2013). But since HA is non-immunogenic, specific antibodies against HA cannot be developed (de la Motte & Drazba 2011). This suggests that the study likely measured some undefined protein attached to the HA molecules that were used for immunisation. A better and more common practice to detect HA is to use a hyaluronic acid binding probe (HABP), a protein that specifically binds the HA backbone. Whatcott et al. used a HABP based method to evaluate the HA expression, but quantified HA expression as the ratio between HA positive staining and total tumour area (Whatcott et al, 2015). This type of method is highly affected by cellularity and rather measures the stromal volume in relation to the amount of cancer cells in a sample, than stromal HA expression.

In summary, while there are several implications that HA promote PDAC, the prognostic relevance of stromal HA expression remains to be elucidated. Additionally, no study has evaluated its potential as a circulating biomarker in PDAC.

5.2 CD44 receptors

The transmembrane receptor cluster of differentiation 44 (CD44) is expressed on virtually all vertebrate cell surfaces. The messenger-RNA (mRNA) encoding the stem of the extracellular domain of CD44 can undergo
alternative splicing, resulting in variant isoforms (CD44v) of the receptor. The variant isoforms have altered ligand binding properties and differ from the standard isoform (CD44s) that lacks all variant exons (Figure 6) (Zoller 2011). In this thesis, CD44s and the variant isoform 6 (CD44v6) are studied.

![Figure 6. The structure of the CD44 receptor and variant isoforms. CD44 is composed of an extracellular, a transmembrane and an intracellular domain. Variations in the extracellular domain result in variant isoforms of the receptor. Adapted from Zöller et al., 2011 © Macmillan Publishers Limited. Used with permission.](image)

5.2.1 CD44 signalling in cancer

CD44 receptors confer cell adhesion and bind stromal ligands to constitute a link between ECM and the intracellular milieu (Zoller 2011). HA is considered the principal CD44-ligand, but both Col IV and OPN, that are described later in this chapter, have also been reported to interact with CD44 (Ishii et al, 1993; Kolb et al, 2005).

In cancer, CD44 signalling acts tumour promoting, as shown in various cell and mouse models (Zoller 2011) (Figure 7). HA initiate CD44 signalling by triggering complex formations between CD44 and growth factor receptors (RTKs), such as ERBB-receptors and the HGF receptor MET that are expressed on cancer cell surfaces. CD44, which lacks intrinsic kinase activity, utilize the kinase domains of these RTKs to transmit signals downstream of MAPK and PI3K pathways. These signalling events promote increased tumour growth, survival and migration (Misra et al, 2003; Ghatak et al, 2005; Meran et al, 2011; Matzke-Ogi et al, 2016).

Another branch of CD44 signalling promotes cell migration by activation of cytoskeletal proteins, such as ankyrin and ezrin (Zhu & Bourguignon 2000; Jung et al, 2011). Moreover, by recruiting ECM degrading matrix metalloproteinases (MMPs), CD44 promote both tissue invasion through the ECM and the release of matrix embedded growth factors (Yu & Stamenkovic 1999; Yu & Stamenkovic 2000).
CD44 receptors are overexpressed in various malignancies and have also been implicated as markers of cancer-initiating cells (CICs). CICs are stem cell-like cancer cells believed to be responsible for tumour relapse and metastasis (Zoller 2011).

5.2.2 CD44 in pancreatic cancer

Excessive CD44-signalling is normally inhibited by the tumour suppressor TP53 (Godar et al, 2008) which is commonly mutated in PDAC (Makohon-Moore & Iacobuzio-Donahue 2016). Apart from p53 loss, high CD44 expression can be induced by the commonly overexpressed ataxia-teleangiectasia group D complementing gene (ATDC). ATDC accelerates PanIN to PDAC transformation in KRAS-driven mouse models, concomitant with an up regulation of CD44s (Wang et al, 2015).

High tumour expression of CD44s and CD44v6 have been associated with poor survival in PDAC (Hong et al, 2009; Lee et al, 2014; Li et al, 2015). In mouse model studies of PDAC, both gemcitabine treatment and chemoradiotherapy selects for a surviving CD44+ cell population. Further, anti-CD44 blocking reduce tumour growth, metastasis and relapse after
oncological therapies, suggesting a role for CD44 in tumour relapse (Lee et al, 2014; Molejon et al, 2015).

Most studies on CD44 and its interactions have been conducted on the CD44 standard isoform (CD44s). However, both CD44s and CD44 variants confer specific functions in cancer. As an example, CD44v6 (but not CD44s) acts as a co-receptor to the HGF receptor MET and inhibiting the CD44-MET interaction reduces tumour growth and metastasis in mouse models of PDAC (Matzke-Ogi et al, 2016) (Figure 7). Similarly, CD44 variants but not CD44s stimulate PDAC cell motility in rats upon interaction with the ECM molecule osteopontin (Katagiri et al, 1999), which is introduced in section 5.5.1 below.

CD44s, on the other hand, has specific functions in EMT, a process that has been shown to depend on a switch from CD44 variants to CD44s in breast cancer (Brown et al, 2011). Similarly, PDAC cells undergo EMT upon induced overexpression of CD44s (Jiang et al, 2015).

5.3 Collagens

Collagens are structural and functional proteins that are abundant in the ECM in healthy and diseased tissues. The desmoplastic PDAC stroma is predominantly rich in fibrillar collagens such as type I and type III collagen (Rasheed et al, 2012). Type I collagen is secreted by PSCs upon stimulation from PDAC cells, and has been shown to stimulate pancreatic cancer cell proliferation and survival in vitro (Armstrong et al, 2004). On the contrary, high expression of fibrillar collagens in human PDAC tumours have been associated with a better prognosis (Erkan et al, 2008). Studies on PDAC desmoplasia have predominantly focused on type I collagen, and less is known about cellular responses and the pathophysiological roles of other types of collagens. This thesis puts focus on the basement membrane proteins type IV collagen (Col IV) and Endostatin, the latter being the cleaving product of type XVIII collagen (Col XVIII), and their interactions with integrin receptors.

5.3.1 Basement membranes

Col IV and Col XVIII are important structural proteins in normal basement membranes (BM). BMs constitute thin and denser parts of the ECM that underly epithelial and endothelial cells, and mediate their structural support and survival signals (Kalluri 2003). Upon tissue invasion and metastasis, cancer cells trespass both epithelial and vascular basement membranes at several sites and propagate through the ECM. A central mechanism in cancer cell invasion is the breakdown of collagens by MMPs, mainly MMP-2 and -9, which facilitate the invasion through these dense tissue networks (Kelley et al, 2014).
5.3.2 Type IV Collagen (Col IV)

Type IV collagen (Col IV) is a sheet-forming collagen that is composed of combinations of six different α-chains, denoted α1(IV) - α6(IV). Each chain is composed of three domains – an N-terminal domain (7s), a triple-helical collagenous domain (CB3) and a non-collagenous C-terminal domain (NC1). The six chains combine into three known combinations (protomers) - α1α1α2, α3α4α5 and α5α5α6. The promomers are secreted into the ECM where they self assemble into a structural scaffold (Figure 8). α1α1α2 is expressed in all BMs, while the other protomers are variably expressed in BMs in certain tissues (Khoshnoodi et al, 2008).

In PDAC, an altered Col IV expression has been suggested; the BM-restricted expression is lost and Col IV tend to accumulate in the tumour stroma (Lee et al, 1994; Linder et al, 2001).

Circulating Col IV has been suggested as a potential biomarker in several cancer forms. As an example, plasma Col IV levels are increased in colorectal cancer liver metastasis, and high levels are associated with poor survival (Nystrom et al, 2011; Rolff et al, 2016). Moreover, a strong Col IV expression is found near the cancer cells in liver metastases irrespective of its primary origin (Burnier et al, 2011). Our research group showed that Col IV is elevated in plasma samples from PDAC patients before surgery, and that persistent high levels at 4 weeks after surgery is associated with poor postoperative survival (Ohlund et al, 2009).
Col IV has a tumour promoting role in vitro by stimulating PDAC cell proliferation and migration in an integrin dependent manner (Grzesiak & Bouvet 2006; Sawai et al, 2008). Moreover, it has been demonstrated that PDAC cells can synthesise Col IV independent of PSC stimuli in vitro (Lohr et al, 1994). However, little is known regarding the function of endogenous Col IV synthesis by PDAC cells.

5.3.3 Type XVIII Collagen and Endostatin
Type XVIII collagen (Col XVIII) is expressed in various types of basement membranes including vascular BMs. It has a c-terminal domain that can be cleaved by MMPs, generating Endostatin, a potent anti-angiogenic molecule (Iozzo 2005). Endostatin has been shown to reduce cancer cell growth and metastasis by inhibiting endothelial cell survival in animal models of several cancers (O'Reilly et al, 1997). In PDAC however, endostatin treatment does not affect tumour growth in orthotopic mouse models (Raut et al, 2004).

PSCs secrete pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) when stimulated by PDAC cells. On the other hand, PSCs increase PDAC cell synthesis of endostatin by increasing MMP-dependent cleavage of Col XVIII. Interestingly, supernatant from PDAC cell-PSC co-culture is predominantly rich in Endostatin and inhibit endothelial cell growth in vitro (Erkan et al, 2009). This suggest that while both pro- and anti-angiogenetic substances are released in PDAC-PSC interactions, the balance is tilted towards anti-angiogenesis. This is in line with the hypoxic state in PDAC tumours and might explain why anti-angiogenetic drugs such as anti-VEGF (bevacizumab) are ineffective for treatment of PDAC patients (Kindler et al, 2010).

In PDAC tissue, Col XVIII expression is shifted towards expression in tumour vessels, coinciding with up regulation of MMPs involved in Endostatin generation. Moreover, circulating levels of Endostatin is increased in PDAC patients compared to healthy controls and the levels are normalised after surgery (Ohlund et al, 2008).

5.4 Integrin receptors
Collagens bind to integrin receptors which are abundantly expressed on the surface of various cell types. Integrins are transmembrane receptors composed of one α- and one β-subunit. 18 α-subunits and 8 β-subunits have been described, resulting in 24 known dimer combinations. Similar to the CD44-receptor, integrins have no intrinsic kinase activity. Integrin relayed signalling therefore depends on interactions with kinases, such as RTKs, focal adhesion kinase (FAK), the proto-oncogene tyrosine-protein kinase (Src) and by recruiting proteins associated with the cytoskeleton. Integrins bound to collagens and other ECM molecules mediate survival signals via
increased PI3K/Akt and decreased p53 activity. On the contrary, unligated integrins mediate apoptotic signalling via the pro-apoptotic protein caspase 8 (Desgroisellier & Cheresh 2010). Both integrin $\alpha_1\beta_1$ and $\alpha_2\beta_1$ has been shown to interact with Col IV in PDAC (Grzesiak & Bouvet 2007) and blocking of the $\beta_1$ subunit inhibits Col IV mediated invasion of PDAC cells \textit{in vitro} (Sawai et al, 2008).

5.5 Matricellular proteins

Matricellular proteins are a group of non-structural proteins that interact with both cell surface receptors and other ECM molecules in cellular and stromal regulation. Family members include periostin, secreted protein acidic and rich in cysteine (SPARC), osteopontin (OPN) and Tenascin C (TNC). All members are in one way or another involved in carcinogenic processes (Wong & Rustgi 2013). This thesis focuses on the matricellular proteins OPN and TNC as potential biomarkers in PDAC.

5.5.1 Osteopontin (OPN)

OPN is highly expressed in the ECM in bone and developing tissues (Shevde & Samant 2014). In various cancer forms, OPN has been implied to promote tumour growth and metastasis, by binding to integrins and CD44 on cancer cells (Shevde & Samant 2014). OPN has been shown to stimulate PDAC cell migration in a CD44-dependent manner \textit{in vitro} (Kolb et al, 2005).

mRNA transcripts of OPN are mainly confined to macrophages within the stroma and are absent in the cancer cells in most carcinomas, including PDAC cells. However, protein levels of OPN is still detected both in the stroma and on tumour cell surfaces, indicating that OPN is produced and secreted by stromal cells and bind to cancer cells (Brown et al, 1994; Koopmann et al, 2004; Sedivy et al, 2005). High tissue expression of OPN has been associated with an unfavourable outcome in breast, lung and gastric cancer, and circulating levels of OPN are increased in several cancer forms (Rudland et al, 2002; Bramwell et al, 2006; Rud et al, 2013; Gu et al, 2016).

OPN has been suggested as a potential blood based biomarker in PDAC, since it is present at significantly higher levels in PDAC patients compared to both healthy individuals and chronic pancreatitis patients (Koopmann et al, 2004; Chen et al, 2010; Poruk et al, 2013). Additionally, a combination of serum OPN, tissue inhibitor of metalloproteinase-1 and Ca 19-9 has been reported to outperform Ca 19-9 in discriminating between these conditions. High serum levels of OPN was associated with poor survival in the same study (Poruk et al, 2013).

However, others report a protective effect of OPN expression in PDAC patients. High expression of OPN in cancer cells was associated with improved postoperative survival in a study analysing >200 PDAC tumours.
Despite mainly expressed in the stroma, the prognostic role of stromal OPN expression has not yet been evaluated in PDAC.

5.5.2 Tenascin C (TNC)

TNC is increasingly expressed in tissues undergoing remodelling, such as inflamed, developing and cancerous tissue and has been shown to stimulate proliferation and migration in several cancer forms (Yoshida et al, 2015).

While absent in normal pancreas, TNC is overexpressed in the PDAC stroma (Juuti et al, 2004) and the expression increases with PDAC progression from early PanIN-lesions (Esposito et al, 2006). Impaired SMAD/TGFβ signalling in PDAC cells was recently shown to induce high stromal tissue tension and an ECM rich in TNC, which collectively accelerates tumour progression in KRAS-driven mouse models (Laklai et al, 2016). TNC has also been shown to inhibit gemcitabine effects on PDAC cells in vitro, by stimulating proliferation and inhibiting apoptosis (Shi et al, 2015).

PDAC patients have increased levels of TNC in the circulation. Balasenthil et al showed that the combination of plasma TNC, tissue factor pathway inhibitor (TFPI) and Ca 19-9 outperformed Ca 19-9 in discriminating PDAC from healthy subjects (Balasenthil et al, 2011).

Chapter 6 – Micro RNAs in pancreatic cancer

Micro-RNAs (miRNAs) are short RNA species of ~22 nt length, that are mainly transcribed from non-protein coding genes. Mature miRNAs regulate gene expression post-transcriptionally by interfering with mRNA stability and translation (Ha & Kim, 2014). miRNAs play important roles in regulation of cell behaviour, tissue homeostasis and development, and they can have oncogenic or tumour suppressive functions in cancer (Di Leva et al, 2014). Pathological miRNA alterations have been documented in cancer tissue, cancer cells and cancer patient blood, indicating the potential use of miRNAs as biomarkers of disease (Hayes et al, 2014). In this chapter, the regulation and function of miRNA in normal physiology and in cancer will be discussed, with emphasis on their potential as diagnostics biomarkers in PDAC.

6.1 miRNA biogenesis and regulation of mRNA levels

The miRNA gene transcription and processing into mature miRNA occurs in multiple steps (Figure 9). The primary transcript (pri-miRNA) is cleaved in the nucleus by the enzyme Drosha. The resulting pre-miRNA is then exported into the cytosol and processed by the enzyme Dicer into mature double stranded miRNA (Ha & Kim, 2014). Mature miRNA single strands are incorporated with the RNA-induced silencing complex (RISC) where they act...
as guides to scan mRNA for complementary sequences (Ha & Kim 2014). Target recognition leads to mRNA destabilisation and translational repression (Guo et al, 2010). Cancer is associated with alterations in the miRNA regulatory machinery, affecting the miRNA expression in tumours (Lin & Gregory 2015).

The miRNA family exerts broad control over gene expression. First, a single miRNA typically represses multiple mRNA targets. Second, multiple miRNAs can repress multiple targets simultaneously, causing synergistic effects. However, miRNAs typically induce relatively modest gene expression changes in terms of amplitude, suggesting that multiple miRNAs cooperate to fine tune the gene expression, rather than acting as individual gene switches (Han et al, 2015).

**Figure 9. A schematic presentation of the miRNA biogenesis.** Whereas mRNA is translated from protein-coding genes in the DNA, miRNAs are encoded from non-protein coding genes. Drosha and Dicer processes pri- and pre-miRNA into mature miRNA strands that repress protein translation by interfering with mRNA in the RISC. Adapted from Koch et al., 2013 © Macmillan Publishers Limited, used with permission.

### 6.2 miRNAs in PDAC tissue

Several studies have showed that the miRNA expression profile (the miRNome) in PDAC is different compared to normal pancreatic tissue and chronic pancreatitis (Bloomston et al, 2007; Lee et al, 2007; Szafranska et al, 2007; Szafranska et al, 2008; Zhang et al, 2009). Although the sample sizes in most of these studies are small, and with conflicting results, commonly identified miRNA alterations include the up regulation of miR-21, -155 and -221 as well as down regulation of miR-34, -217 and -375 (Ma et al, 2013). These miRNAs and several other miRNAs are known as oncogenes and tumour suppressors (Di Leva et al, 2014).
miRNA alterations occur already in precursor lesions that precede PDAC. The expression levels of miRNAs are altered in PanIN lesions in both human tissue samples and in KRAS driven PDAC mouse models (Yu et al, 2012; Ryu et al, 2010; du Rieu et al, 2010; LaConti et al, 2011). Similarly, the IPMN miRNome differ between normal tissue, other cystic tumours and PDAC (Habbe et al, 2009; Lubezky et al, 2013) and has been suggested to aid in the differentiation between benign vs malignant IPMNs (Permuth-Wey et al, 2015).

6.3 miRNAs in the circulation

miRNAs circulate in blood bound to the argonaute 2 protein (Ago2) or lipoproteins. Additionally, a small fraction of miRNAs are exclusively contained within exosomes (Valadi et al, 2007; Arroyo et al, 2011; Vickers et al, 2011). Circulating miRNAs are remarkably stable, owing to Ago2 and exosome carriers that protect the intrinsically sensitive species against enzymatic degradation and temperature changes (Valadi et al, 2007; Mitchell et al, 2008). In mouse xenograft studies, cancer cells release human miRNA into the circulation (Mitchell et al, 2008), and cellular uptake of lipoprotein-associated miRNAs with targeting capabilities have been demonstrated in vitro (Vickers et al, 2011). In theory, the release of miRNAs thus leads to a tumour fingerprint in blood samples, conferring a “liquid biopsy” of the tumour.

6.3.1 Circulating miRNAs in PDAC

Circulating miRNA have been proposed as potential diagnostic biomarkers in various cancer forms, including PDAC (Jarry et al, 2014). Most studies of PDAC have selectively assayed miRNAs that are overexpressed in the tumour tissue, to confirm mirroring alterations in circulation (Wang et al, 2009; Ho et al, 2010; Li et al, 2010; LaConti et al, 2011; Liu et al, 2011; Morimura et al, 2011; Kawaguchi et al, 2013; Chen et al, 2014; Abue et al, 2015; Kojima et al, 2015). However, assaying a small subset of miRNAs can lead to a selection bias, leaving important miRNAs undiscovered. Additionally, the majority of these studies had small sample sizes and the findings were not validated in additional cohorts.

Three studies have screened and validated multiple miRNAs in large cohorts. First, Liu et al developed a 7 miRNA panel by measuring serum miR-20a, -21, 24, -25, -99a, -186 and -191, and showed that this panel outperformed the diagnostic accuracy of Ca 19-9. When applied prospectively by following 55 cases of suspected PDAC, the panel correctly identified 83.6% of the patients later diagnosed with PDAC (Liu et al, 2012). Second, Schultz et al performed the largest study so far, by profiling >700 miRNAs in whole-blood samples from >400 patients with PDAC. The miRNA levels were compared with chronic pancreatitis patients and healthy
individuals. From 38 miRNAs dysregulated in their screening set, they finally constructed and validated two panels, comprised of four and ten miRNAs, respectively. Both panels reached accuracy over 80% for correct classification, and the four miRNA panel reached 98% specificity at 85% sensitivity when combined with Ca 19-9 (Schultz et al, 2014). Third, a recent multicentre study found 13 dysregulated miRNAs in PDAC plasma samples, when comparing PDAC to chronic pancreatitis, other pancreas tumours and to normal controls. They concluded that miR-486 was comparable to Ca 19-9 in differentiating between PDAC and normal tissue, but found no plasma miRNA that accurately differentiated PDAC from chronic pancreatitis or other pancreatic tumours (Xu et al, 2016).

While promising, all these studies have only included patients with manifest PDAC symptoms, but a diagnosis at this stage is almost always too late. Thus, the challenge is to find altered miRNA profiles in asymptomatic patients, when cure is still possible.
AIMS

Overall aim

The overall aim of this thesis is to find potential diagnostic and prognostic biomarkers derived from the tumour stroma and from within the miRNA family, to be used in the clinical management of pancreatic ductal adenocarcinoma.

Specific aims

- To characterise the expression patterns of stromal components in PDAC tumours. Specifically, BM proteins (Col IV and Endostatin), matricellular proteins (OPN and TNC), and HA

- To characterise the expression patterns of receptors for stromal components in PDAC tumours. Specifically, CD44 receptors and Col IV-binding integrin receptors

  (Paper I, II and III)

- To determine how Col IV affects PDAC cells *in vitro*

  (Paper I)

- To find stroma-derived, non-invasive, diagnostic and/or prognostic biomarkers for PDAC and compare them with the conventional tumour markers Ca 19-9, Ca 125, TPS and CEA

  (Paper II & III)

- To find prognostic tissue biomarkers within the network of CD44-stroma interactions

  (Paper III)

- To find plasma miRNAs that are altered in PDAC patients

- To determine if plasma miRNAs can be used for early detection of PDAC

  (Paper IV)
MATERIALS AND METHODS

Chapter 7 - Patient and control samples

7.1 Ethical statement
All studies within this thesis were conducted according to the ethical standards of the Helsinki declaration of 1975. All included individuals gave their written informed consent. The studies were approved by the ethical committee of northern Sweden (Dnr 09-175M/2009-1378-31 and Dnr 09-125M/2012-376-32M).

7.2 Cohorts
Normal pancreas and PDAC tissue studies were conducted on two tissue types; (1) tumour tissue that was collected during surgery, snap frozen in liquid nitrogen and long-term stored in -80 °C, and (2) formalin fixed, paraffin embedded tissue (FFPE) retrieved from the medical biobank (Biobanken Norr) at Västerbotten County Council (VLL).

The blood samples analysed in paper II, III and in the miRNA screening phase of study IV were collected before and after surgery at the Department of Surgery, Umeå University Hospital and were long-term stored at -80°C. Control samples were collected from individuals without previous malignant disease, that underwent an endoscopic procedure without pathological findings or from patients who underwent surgery for non-malignant conditions.

In paper IV, plasma samples collected months to years before the time of PDAC diagnosis were used for miRNA analysis. These samples were retrieved from a biobank associated with the Västerbotten Intervention Programme (VIP). VIP is a public health initiative that was launched in 1985 aiming to reduce cardiovascular mortality and morbidity in Västerbotten, Sweden. All inhabitants are offered health examinations at the ages 40, 50 and 60. Participants are also asked to donate plasma samples for future research (Norberg et al, 2010). Within this cohort we identified individuals that were diagnosed with pancreatic cancer between 1990 – 2009 (cases). Cases were matched with healthy VIP participants (controls) by gender, age and sample collection date.

The number of patients and controls included in each study is listed in Table 3.
### Table 3. The number of patients included in the studies

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Blood</th>
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<tbody>
<tr>
<td>PDAC</td>
<td>Controls</td>
</tr>
<tr>
<td>Paper I</td>
<td>9</td>
</tr>
<tr>
<td>Paper II</td>
<td>8</td>
</tr>
<tr>
<td>Paper III</td>
<td>69</td>
</tr>
<tr>
<td>Paper IV*</td>
<td>-</td>
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</tbody>
</table>

* = two cohorts studied

## Chapter 8 - Cell culture experiments

Tumour-promoting effects of Col IV were studied by modifying the environmental conditions for the well-characterized PDAC cell lines HPAC and CFPAC-1 in paper I. The following experiments were performed: (1) cell growth on plastic wells coated with Col IV, Col I or bovine serum albumin (BSA), (2) antibody blocking of the NC1, 7s or CB3 domains of Col IV (3) antibody blocking of Col IV binding integrin receptors and (4) post-transcriptional repression of the Col IV α1 chain gene by RNA interference (Figure 10). RNA interference was performed by transfecting cells with small interfering RNA (siRNA) directed towards COL4A1 mRNA. The siRNA efficacy was validated both at the mRNA and protein level with semi-quantitative real-time polymerase chain reaction (PCR) and immunofluorescence stainings.

![Figure 10. Col IV studies on PDAC cells. Growth conditions were modified by (1) matrix coating with Col IV vs. Col I vs. BSA, (2) blocking different Col IV domains, (3) integrin blocking and (4) siRNA transfection](image-url)
Effect on cell proliferation was studied using a luminescence-based cell viability assay (Cell-titer Glo). In this assay, cellular adenosine triphosphate (ATP) will convert added luciferin to luciferase and enable ATP quantitation. ATP levels correlate with the number of metabolically active cells. Additionally, the fraction of cells in S-phase was measured with flow cytometry. Effects on apoptosis were studied by measuring a caspase-cleaved fragment of cytokeratin 18 with an M30 antibody enzyme-linked immunosorbent assay (ELISA), which specifically detects epithelial cell apoptosis. Effects on migration were studied with a wound healing assay.

Chapter 9 – Tissue studies and staining methods

9.1 Tissue microarray (TMA) construction

A tissue microarray was constructed and used for immunohistochemical staining procedures (IHC) in paper III. Tumour areas were selected and cores (1 mm in diameter) were drilled from FFPE tumour tissue. Cores from multiple patients were collected onto a single paraffin embedded tissue block (Figure 11).

Patients that underwent pancreatic surgery between 1990 – 2009 at the Department of Surgery of Umeå University Hospital, with a PDAC diagnosis, were included in the TMA. Together with an experienced pathologist, three areas for drilling were selected from each primary tumour and 1-3 areas were selected from metastatic lymph node specimens. Cores were drilled with a TMA Grand master machine and placed randomly on a common block (recipient block).

The main benefits of TMA:s are: (1) the staining procedures are performed on the same slide, which minimises technical variance, (2) it facilitates high-throughput IHC staining and analysis (3) the areas for analysis are predefined, which minimises observer bias. The main drawback of using TMA is that less tissue volume is analysed, and tissue heterogeneity may be underestimated. However, tissue biomarker expressions on TMA cores typically correspond well with expression on whole sections (Bentzen et al, 2008).
Figure 11. TMA construction. Cores from multiple tumour specimens (donor blocks) were collected in a recipient paraffin block – the TMA. The TMA was then sectioned and used for the IHC procedures in paper III.

9.2 Tissue and cell stainings

TMA blocks were sectioned and stained for CD44s, CD44v6, Col IV and OPN using routine antibody-based IHC protocols (Paper III). HA was stained using a biotinylated HA binding protein (HABP), which specifically binds to the HA backbone, instead of using antibodies. The biotin molecule binds an avidin-peroxidase complex and the peroxidase enzyme oxidates 3,3′diaminobenzidinetetrahydrochloride (DAB). This results in a brown colour development, visible in regular bright field microscopy (Paper III). The stainings were used to semi-quantify expression levels in tissue (described in detail in the methods section in Paper III). Immunoflourescent stainings was performed on cells and frozen tissue sections in Paper I to visualise the expression of Col IV, Col I, integrin receptors and endothelial cells (CD31), and in Paper II to visualise the expression of Col IV, Endostatin, OPN, TNC, Ca 19-9, CEA and Ca 125.

A proximity ligation assay (PLA) was used in paper III to visualise proximity between CD44 and stromal ligands (OPN and Col IV). The technique utilizes PLA probes, secondary antibodies conjugated with linear DNA fragments, which are ligated when their respective antibodies bind in close proximity (30-40 nm), indicative of interactions. Probe ligation result in formation of a circular, single stranded DNA molecule. Amplification of the DNA molecule with a DNA polymerase results in formation of a long DNA product attached to one of the probes. A peroxidase labelled oligonucleotide that binds to this DNA product subsequently allows conversion of a substrate (NovaRED) into a brown colour.
Chapter 10 – Analysis of blood samples

10.1 Measurements of circulating stromal components
Enzyme-linked immunosorbent assays (ELISA) was used to measure circulating levels of Col IV, Endostatin, TNC C, OPN, TPS and HA (Paper II-III). A multiplex bead assay was used to measure Ca 19-9, CEA and Ca 125 (Paper II & IV).

The ELISA assays used were mainly “sandwich” ELISA:s. In a sandwich ELISA, the protein that is to be measured (the antigen) is bound to the bottom of each multiplate well by pre-coated antibodies that bind to an epitope on the antigen. Secondary antibodies are then added, that bind to another epitope on the antigen. The secondary antibodies are conjugated with an enzyme that converts a substrate (commonly chromogen), to produce a colour reaction that is positively correlated with the amount of protein in the sample.

Serum HA levels were measured with two different ELISA-like assays based on HABP binding instead of antibodies: one sandwich assay (Corgenix, USA), and one competitive assay (Eschelon Biosciences, USA) (Paper III). In the competitive assay, serum HA is first allowed to bind enzyme-conjugated HABP. The mix is then added to wells with pre-coated HA molecules. Enzyme-conjugated HABP not bound to serum HA in the first step, will bind to HA in the wells in the second step. The signal read will thus be inversely correlated with the amount of HA in the serum sample.

Haselrodt et al. compared the performance of these assays using known HA concentrations of different molecular mass (Haserodt et al, 2011). They showed that the sandwich assay (Corgenix) has less intra- and inter-assay variability compared to the competitive assay (Eschelon), but that the sandwich assay has less sensitivity for low M HA (<27 kDa).

Multiplex bead ELISAs utilize magnetic beads coated with multiple antibodies directed at different antigens, which permit measurements of several protein concentrations simultaneously. The antigens bind to antibodies on the beads, which are kept in the wells magnetically. The secondary antibodies emit light at different wavelengths, which can be separated with a spectrophotometer and allow for multiple antigens to be quantified simultaneously.

10.2 Measurements of circulating miRNAs

10.2.1 Plasma miRNA isolation
miRNA was extracted using a phenol-chloroform-based protocol (Paper IV). Briefly, the phenol-chloroform mixture allows phase separation of RNA from DNA and proteins. The upper phase, that contains RNA species, was purified using ethanol washing steps through spin columns, and was finally eluted in
RNase free water. Careful sample handling and an RNase-free lab environment was strived for through all steps.

miRNA isolation from blood samples has some considerations compared to RNA isolation from tissues and cells. The RNA amount in biofluids is too small to allow for accurate RNA yield measurements using spectrophotometry (Kroh et al, 2010). Therefore, fixed volumes of plasma were isolated and fixed volumes of eluted miRNA were used in subsequent quantification. Additionally, synthetic “spike-in” RNAs were added to control for isolation efficacy.

Since certain miRNAs are enriched in red blood cells, miRNA levels are affected by haemolysis in plasma samples (Kirschner et al, 2013). Haemolysis was controlled for by measuring plasma haemoglobin levels spectrophotometrically, based on the formula $Hb = (154.7 \times \text{the absorbance at 415 nm}) - (130.7 \times \text{the absorbance at 450 nm}) - (123.0 \times \text{the absorbance at 700 nm})$ (Fairbanks et al, 1992).

10.2.2 Plasma miRNA measurements
Eluted miRNAs were subjected to reverse transcription PCR, to synthesise complementary DNA (cDNA). cDNA was then amplified and quantified using real-time quantitative PCR (RT qPCR). Here, the principles of the RT qPCR assay used in paper IV will be briefly described. The assay uses a fluorescent reporter (SYBR green) that emits a fluorescent signal when bound to double stranded DNA. First, miRNA-specific primers anneal to the cDNA and a DNA polymerase syntheses double stranded DNA products that emit a SYBR green signal. The PCR is repeated in multiple cycles resulting in an exponentially increased product amplification and signal strength. Eventually, a PCR cycle will generate a detectable signal, called the cycle threshold (Ct). Abundant cDNAs will generate detectable signals in earlier cycles (low Ct values) than less abundant cDNAs (high Ct values) and this correlation is used to quantify the input amount of specific miRNAs.

Importantly, this method yields relative differences between miRNA levels within a sample, rather than exact miRNA concentrations. Hence, to compare samples, the miRNA levels must be normalised against a background variable. Various methods to normalise the data have been described, and there is no consensus favouring a certain normalisation method. Commonly applied methods include the use of reference miRNAs that are commonly stable in both health and disease (house-keeping genes), measuring added spike-in RNAs or by normalising for the total expression of miRNAs (Mestdagh et al, 2009; Meyer et al, 2010). The latter was used for normalisation in paper IV. Methods for normalisation vary between miRNA studies, which to some extent may explain differences in the final results.
Of note, the miRNA measurements used in paper IV were sent to Denmark and executed by Exiqon Services (Exiqon, Denmark). However, I performed all quantification steps similarly in pilot experiments.

Mesdagh et al compared 12 commercially available miRNA quantification platforms, and the Exiqon platform performed among the top three in terms of assay reproducibility, accuracy, sensitivity and specificity (Mesdagh et al, 2014).

Chapter 11 – Statistics

11.1. Univariate statistics
For independent group wise comparisons of continuous variables, students t-test or Wilcoxon rank sum test (Mann-Whitney) was performed. Wilcoxon signed rank test was used for paired comparisons. When more than two groups were compared, analysis of variance (ANOVA) or Kruskal-Wallis test was used, followed by post-hoc Bonferroni’s correction of the p-value. Categorical data were compared with Chi-Square test or Fischer’s exact test. Survival analyses were performed with Kaplan-Meier estimates, log-rank tests, Wilcoxon-Breslow tests and Cox proportional hazard models. Correlations were calculated with Spearman’s rank correlation. Area under the curve (AUC) for biomarker accuracy was calculated with receiver operating characteristics (ROC) curves.

11.2. Multivariate statistics
Multiple miRNA levels were combined into a multivariate model in Paper IV with the aim to separate patients from controls. This was performed using the multivariate statistical method orthogonal projections to latent structures (OPLS). Specifically, OPLS for discriminative analysis (OPLS-DA) was used, where the response variable is binary (Bylesjö et al, 2006). In a multivariate data set, variances in the variables/miRNA levels, might depend on whether an observed individual is a patient or a control. However, the variables will also depend on other known and unknown variation (such as age and gender), or technical variation introduced in the measurements (non-related factors). OPLS aims to separate variance that depend on group designation (the predictive component) from non-related variation (orthogonal components). The final model generates a prediction, or estimate of the response, for each observation. The prediction uses the score value for the predictive component (the value of each observation on the x-axis in figure 12). In an ideal model, predictions are 0 for all controls and 1 for all patients. The main benefits of OPLS are (1) that it uses multiple variables to get a strong separation between patients and controls, and (2) that it separates predictive from non-predictive information (Figure 12).
Figure 12. The OPLS-DA model separates variance related to group designation from non-related (orthogonal) variation. In this example, each dot represents an individual. The two groups cluster on each side on the Y-axis. A) represents the variation correlated with group designation (predictive component). B-C) represent variations that are not correlated with group designation (orthogonal components). The OPLS model removes parts of the non-related variation by adding orthogonal components, to improve the model (better separation between cases and controls).

However, OPLS carries a significant risk of model overfitting. Overfitting refers to the construction of a model that does not perform well in an independent cohort. The risk of overfitting increases for each orthogonal component that is added to the model. This can be controlled for by using a cross-validation procedure (Figure 13). One seventh of the sample set is excluded and the model is fitted for the remaining 6/7 using the same number of components. Finally, the responses of the excluded observations are predicted (patient or control?) using the resulting model. This is repeated seven times in order to predict all individuals. A good model will have high accuracy for the cross-validated predictions, which can be estimated with a significance testing procedure called CV-ANOVA (Eriksson et al, 2008).

Figure 13. Cross validation to control for model overfitting. For each included component, the explanatory value (response) of the group designation is increased. Cross validation will confirm increased model performance initially, but reaches a maximum. Eventually, the explained variation using cross-validated prediction will be decreased when too many components are added to the model.
RESULTS

Chapter 12 - Expression patterns of stromal components and their receptors in normal pancreas and PDAC tissue

12.1 Expression patterns of stromal components are altered in PDAC

12.1.1 Expression of Col IV and Endostatin
In normal pancreatic tissue, both Col IV and Endostatin was expressed in acinar, ductal and vascular basement membranes (BM) (Figure 14).

In the PDAC TMA, Col IV was expressed close to the cancer cells, forming BM-like structures in most primary tumours (69 %) and over one third of the lymph node metastases (38 %) (Paper III). There was no correlation between expression in BM-like structures and tumour differentiation grade (Paper III, suppl. table 1). Col IV was also expressed in the tumour stroma surrounding cancer cell clusters in most primary tumours (73 %) (Paper III). Both the BM-like and the stromal expression varied among the patients from total absence to a strong expression, and the expression in the two compartments were strongly correlated (rho = 0.85, p<0.0001) (Figure 15 and Paper III, suppl. figure 2). Additionally, Col IV was expressed on the surface of monocultured PDAC cells, verifying that PDAC cells synthesise Col IV in vitro (Paper I, figure 3).

Endostatin was expressed in BM-like structures and weakly in the surrounding stroma in highly and moderately differentiated PDAC tumours. In poorly differentiated tumours, Endostatin was mainly expressed in vascular basement membranes (Paper II, figure 1).

![Figure 14. The expression of Col IV, Endostatin, OPN and TNC in normal pancreas tissue (red). The stromal components are counterstained with CK18 (green) that stains the cytoskeleton in cells of epithelial origin. Cell nuclei are stained blue (DAPI). Scale bar = 25 µm. Corresponding stainings of PDAC tissue is found in paper II, figure 1. The legend panel indicate tissue compartments in the IF micrographs.](image-url)
12.1.2 Expression of the matricellular proteins OPN and TNC
In normal pancreatic tissue, TNC was absent, while OPN stained focally in ECM interspacing clusters of acini (Figure 14). All PDAC tissue samples stained strongly for TNC in the stroma, and OPN was expressed in both cancer cells and in the surrounding stroma (Paper II, figure 1). In the TMA, stromal OPN expression was expressed in 93 % of the primary tumours and 75 % of the metastatic lymph nodes, and the expression varied from absent/weak to strong (Figure 15).

12.1.3 Expression of HA
In normal pancreatic tissue, HA was sparsely expressed in ductal connective tissue (Paper III, figure 2).
In the vast majority (79 %) of the primary tumours and all metastatic lymph nodes in the TMA, a strong and widely distributed stromal HA expression was found both close to the cancer cells and in the surrounding stroma (Figure 15).

![Figure 15. Expression of stromal components and CD44 receptors in PDAC (TMA). Weak and strong expression respectively. Scalebar = 25 µm.](image)

12.2 Expression patterns of integrin and CD44 receptors are altered in PDAC

12.2.1 Expression of integrin receptors
In normal pancreatic tissue, the integrin subunits α2 and β1 were expressed on the basolateral surface of ductal epithelium. The α1 subunit was exclusively expressed on endothelial cells, indicated by co-expression with the endothelial cell marker CD31 (Paper I, figure 1-2).
In PDAC, both integrin α2 and β1 were expressed over the entire cancer cell surfaces, while integrin α1 was expressed sporadically in poorly differentiated PDAC tumours (Paper I, figure 2C). All three integrin subunits co-localized with Col IV in PDAC, indicating interactions (Paper I,
In PDAC, both receptors were expressed on cancer cell surfaces in most tumours, but the percentage positive PDAC cells varied among tumours examined (Figure 15). CD44s co-localised with Col IV in vitro (Figure 16). However, the PLA stainings could not detect any interactions between either Col IV and CD44s nor OPN and CD44s near PDAC cells in tissue specimens. Only sporadic interactions were noted between CD44s and both ligands in the stroma, in a subset of the tumours examined (Paper III, figure 3).

Chapter 13 - Effects of Col IV in PDAC in vitro

13.1 Exogenous Col IV stimulates PDAC cell proliferation, migration and survival in vitro

To study the cellular effects of Col IV, PDAC cells were grown in wells coated with Col IV, Col I or BSA. Both Col I and Col IV stimulated cell proliferation compared to BSA, in a dose-dependent manner (Paper I, Figure 4B). Moreover, Col IV, but not Col I, significantly inhibited PDAC cell apoptosis (Paper I, Figure 4C). Cytoplasmic protrusions were noted on cells grown on both Col I and Col IV compared to BSA, indicative of migratory phenotypes (Figure 17). In wound healing experiments, a faster wound closure was demonstrated when cells were grown on Col I and IV compared to BSA (Paper I, Figure 4D).
13.2 Endogenous Col IV stimulate PDAC cell proliferation, migration and survival in vitro

Since PDAC cells synthesise Col IV in vitro, it was hypothesised that endogenous Col IV may be involved in a tumour-promoting autocrine loop. To test this hypothesis, RNA interference and blocking studies were conducted. First, monoclonal antibodies were used to block Col IV binding integrin subunits α1, α2 and β1 on PDAC cells, which inhibited proliferation in all blocking experiments (Paper I, Figure 5A). Second, antibodies were used to block domains on the Col IV α1 chain. Blocking of the CB3 domain reduced proliferation, while proliferation was unaffected when the 7s or the NC1 domains were blocked (Paper I, Figure 5B). Third, the gene expression of the Col IV α1-chain (α1(IV)) was reduced by siRNA transfection. Reduction of α1(IV) was verified both at the mRNA (0.45 fold decrease) and protein level (Paper I, Fig 5C-1). α1(IV) transfection inhibited proliferation, evidenced by both reduced viability (20% less) and a reduced fraction of cells in S-phase (28.6 vs. 22.5 %) (Paper I, figure 5C-2). Additionally, the α1(IV)-transfected cells migrated slower in the wound healing assay (Paper I, figure 5C-3), and apoptosis was increased compared to the control-transfected cells (Paper I, figure 5C-4). The reduced proliferation caused by α1(IV)-transfection was rescued when cells were allowed to grow on Col IV coated plates. The rescue failed when the integrin subunit β1 was blocked, evidenced by a dramatic decrease in cell viability (Paper I, figure 5D).

Chapter 14 – Biomarkers in pancreatic cancer

14.1 CD44s, CD44v6 and OPN predict PDAC prognosis

Cell counting and semi-quantification was performed to score the expression of CD44, CD44v6, Col IV, OPN and HA in the TMA. The association with postoperative survival was assessed using Kaplan-Meier (K-M) estimates and Cox regression.

High expression of both CD44s and CD44v6 were associated with an unfavourable postoperative outcome in K-M estimates (Paper III, figure
The median survival was 8 vs. 16 months for high vs. low CD44s (p=0.005) and 8 vs. 14 for high vs. low CD44v6 (p=0.04). In multivariable Cox models, high CD44s expression independently predicted poor postoperative survival (hazard ratio [HR]: 1.9, p = 0.015, adjusted for age, gender, tumour grade TNM stage, non-radical resection and oncological treatment) (Paper III, table 3). On the contrary, high stromal OPN expression was associated with prolonged survival in K-M estimates (median survival 17 vs. 10 months, p = 0.03) and was an independent predictor of better outcome in Cox models (HR: 0.8, p = 0.046) (Paper III figure 2 & table 3).

Neither the expression of HA nor Col IV in tumour tissue were significantly associated with postoperative prognosis. However, a trend towards improved survival was noted with high Col IV expression in both the surrounding stroma (15.6 vs. 10 months, p = 0.2) and BM-like structures (15 vs. 10 months, p = 0.4) in primary tumours and lymph node metastases (17 vs. 6.7 months, p = 0.06) (Paper III, figure 2).

14.2. Stromal components as circulating biomarkers in PDAC

14.2.1 Circulating stroma-derived markers are elevated in PDAC patients

Our group previously showed that circulating levels of Col IV and Endostatin are elevated in PDAC patients compared to healthy individuals (Ohlund et al, 2008; Ohlund et al, 2009). Similarly, other groups have shown that circulating OPN and TNC are potential stroma-derived tumour markers of PDAC (Koopmann et al, 2004; Chen et al, 2010; Balasenthil et al, 2011; Poruk et al, 2013). Supposedly, these stroma-derived markers reflect the extent of PDAC desmoplasia in tumours, while conventional biomarkers reflect aspects of cancer cells. The idea was that combining these two marker types might reflect both these elements in PDAC pathology.

To evaluate the potential of these stroma-derived proteins as diagnostic and prognostic markers, they were compared with with conventional tumour markers that were confirmed to be expressed by cancer cells (Ca 19-9, Ca 125, TPS and CEA) (Paper II, figure 1). Circulating levels of the eight markers were measured in PDAC patients, both pre- and postoperatively, and compared to healthy controls. The circulating levels of the stroma-derived and conventional biomarkers in the three groups are summarised in Paper II, figure 2.

All measured stromal-derived markers and all conventional markers, except CEA, were significantly increased in PDAC patients in the preoperative setting. After surgery, Endostatin and Ca 19-9 decreased to similar levels as the controls. TPS levels decreased significantly but were still higher compared to the controls. The remaining markers remained elevated at follow-up (≥28 days after surgery). Significant correlations were found
between circulating levels of Ca 19-9 and Ca 125, between TNC and Ca 125 and between CEA and OPN. This suggests that these markers are affected by similar tumour associated or systemic changes, reducing the likelihood that combining these markers will improve diagnostics. No correlations were found between Col IV, Endostatin or TPS levels and the other markers, suggesting that the levels of these markers reflect different tumour processes. In addition, less variation within the groups were noted for the stroma-derived markers compared to conventional biomarkers. None of the stroma derived markers were correlated with bilirubin levels or other liver lab tests (Paper II).

A clinical situation was simulated to evaluate combining stromal and cancer cell derived biomarkers (Paper II, table 3). A cut-off level for each marker was set to the 95th percentile of control samples, to divide patient measured levels of each marker into normal and increased. Most PDAC patients (75%) presented with increased levels of 4/8 markers or more. One patient presented with only increased OPN levels. Two patients presented with unmeasurable Ca 19-9 levels and these patients had increased levels of at least one stromal marker. Of note, the cut-off used equals fixing the specificity level at 95%. The sensitivities observed at this level of specificity were: 58% for Col IV and Endostatin, 33% for TNC and 75% for OPN. Corresponding sensitivities for conventional biomarkers were: 83% for TPS and Ca 19-9, 58% for Ca 125 and 17% for CEA.

14.2.2 Circulating levels of HA are increased in PDAC patients
Since HA was expressed in almost all primary tumours and metastatic lymph nodes, it was hypothesised that this might be reflected in the circulation. Serum levels of HA (S-HA) were measured with two different ELISA-like assays; one sandwich assay, and one competitive assay. The results are summarised in Paper III, figure 4.

PDAC patients had significantly elevated levels of S-HA compared to healthy controls (p≤0.0001), both in the pre- and postoperative setting. The area under the curve (AUC) in ROC analysis was 0.84 for S-HA, independent of the measurement method used. The sensitivity for discriminating PDAC patients from controls was slightly higher using the sandwich assay (sensitivity/specificity 85%/76%), but the specificity was lower compared to the competitive assay (sensitivity/specificity 79%/82%) (Paper III, figure 4A-D).

To challenge these findings, potential confounders were investigated. S-HA increased with increasing age in the control groups (rho = 0.4-0.5) (Paper III, figure 4E-F) and was suspected to confound the results because of a significant age difference between patients and controls (p = 0.005) (Paper III, table 2). Ordinal regression models showed that S-HA levels still differed significantly between patients and controls after adjusting
for age (p<0.0001) (Paper III, supplementary table 4A). However, the relation between S-HA and age was different in patients compared to controls when measured with the competitive assay; while S-HA increased with age in the control group, it did not in PDAC patients (Paper III, supplementary table 4B). As a consequence, regression lines intersected in a way that S-HA measured with the competitive assay did not permit age adjustment in elderly (>80 years of age) (Paper III, supplementary figure 3). On the contrary, S-HA measured with the sandwich assay permitted age adjustments at all ages. Moreover, S-HA was positively correlated with serum bilirubin levels in both assays (sandwich: rho = 0.35, competitive: rho = 0.5) (paper III, figure 4G-H).

14.2.3 Circulating stromal components carry prognostic information
Higher plasma levels of Endostatin was associated with shorter survival in both the preoperative (HR: 1.2, 95% CI 1.02-1.48) and postoperative (HR: 1.1, 95% CI 1.00-1.15) setting (adjusted for age and gender). An association with worse prognosis was found with higher postoperative plasma levels of OPN (HR: 1.5, 95% CI 1.11 – 2.03), Col IV (HR: 1.1, 95% CI 1.01-1.18) and Ca 125 (HR: 1.1, 95% CI 1.02 – 1.27) (adjusted for age and gender). No significant associations with survival were found for Ca 19-9, CEA, or TPS (Paper II).

Higher preoperative S-HA predicted shorter survival independent of TNM stage, tumour grade, age, gender and oncological treatment (HR: 1.3, 95% CI 1.02 – 1.77) (Paper III, table 4).

14.3. Plasma miRNAs are altered at PDAC diagnosis but the alterations appear late
Several studies have highlighted the potential use of circulating miRNAs as diagnostic markers for PDAC (Schultz et al, 2014; Srivastava et al, 2014; Xu et al, 2016). In addition, miRNA alterations are present in precursor lesions that precede PDAC (Habbe et al, 2009; Yu et al, 2012). Hence, it was hypothesised that plasma miRNAs alter early in the disease course and are potential biomarkers for early PDAC discovery. This was evaluated in a two-step process.

First, miRNA alterations present at the time of diagnosis were determined. The plasma levels of 372 miRNAs were measured in preoperative plasma samples from 23 patients with stage I-II PDAC, and compared to 22 healthy controls. Fifteen miRNAs were significantly altered at this stage, and were selected as miRNA candidates. Specifically, plasma levels of let-7d-3p, miR-22-3p, -24-3p, -34a-3p, -122-5p, -130b-3p, -197-3p, -423-3p, -574-3p, and -885-5p were increased and miR-26a-5p, -101-3p, -106-5p, -144-3p and -451a were decreased in PDAC patients (Paper IV, table 3). A multivariable statistical model was constructed that combined
the candidate miRNAs, using OPLS-DA. The cross-validated model was highly significant ($p<0.0001$), indicative of a strong model and a low risk of over fitting. The 15 miRNA combination model outperformed Ca 19-9 in ROC analysis (AUC: 0.96 vs. 0.92) (**Paper IV, figure 2B & 3B**).

Next, the candidate miRNAs were measured in plasma samples collected before PDAC diagnosis, using samples associated with the Västerbotten Intervention Programme (VIP). Prediagnostic plasma samples collected from 67 patients that later developed PDAC were analysed along with 132 matched, healthy controls. In this cohort, none of the candidate miRNAs were significantly altered (**Paper IV, table 4**). The cohort was stratified into time intervals in relation to the diagnosis date to analyse changes over time. Again, no significant changes were found, neither for individual miRNAs, nor with the OPLS-DA model (**Paper IV, figure 2A**). On the contrary, Ca 19-9 was significantly increased in samples collected less than 5 years before diagnosis ($p = 0.044$) (**Paper IV, figure 2C**). However, ROC analysis revealed a poor discriminative performance for both Ca 19-9 and the miRNA combination in this time span (**Paper IV, figure 3A**).
DISCUSSION

The results in this thesis demonstrate that several molecules involved in cancer cell-stroma interactions confer prognostic information and are potential biomarkers in PDAC. The BM protein Col IV has a tumour promoting role in vitro, and the results of knocking down endogenous Col IV suggest a newly discovered tumour promoting autocrine loop. Finally, while plasma miRNAs are significantly altered at the time of PDAC diagnosis, the late appearance of these changes in the disease course suggest a limited use for plasma miRNAs in early PDAC detection.

Chapter 15 – Functional roles of Col IV and integrins

Malignant cancer cells detach from the BM when they invade through the underlying stroma and metastasise to distant organs (Kalluri 2003). Since this involves disruption of integrin-BM interactions, the cancer cells must utilize mechanisms to either retain or become independent of the survival signals that are mediated by this interaction. This thesis present clues to such a mechanism.

The results presented here indicate that malignantly transformed pancreatic cells continue to depend on integrin-ECM interactions. Integrin α1, α2 and β1 expression was retained on PDAC cells, and proliferation was reduced when these integrins were blocked in vitro. The expression patterns of integrin subunits α2 and β1 were shifted from a basolateral expression in the normal pancreas, towards a diffuse expression covering the entire cell membrane in PDAC tissue. This potentially facilitates integrin-ECM interactions when PDAC cells invade through stromal tissue. Moreover, PDAC cells contribute to Col IV synthesis, which in turn stimulate their proliferation, migration and survival. These cellular effects were shown to be dependent of the CB3 domain of Col IV and integrin subunit β1.

Taken together, the findings suggest that PDAC cells synthesise Col IV to stimulate their proliferation, migration and survival via an autocrine loop, and that the effect on proliferation is, at least in part, dependent of the CB3 domain on the Col IV α1-chain and integrin subunit β1.

Here, Col I also stimulated proliferation and migration, verifying previous reports (Grzesiak & Bouvet 2006). However, most PDAC cell lines do not synthesise Col I (Lohr et al, 1994). Instead, Col I synthesis has been shown to be dependent on cancer cell-pancreatic stellate cell (PSC) interactions (Bachem et al, 2005; Pothula et al, 2016).

The findings presented here also suggest that integrin blocking is a potential treatment strategy in PDAC, which is supported by previous in
vitro and in vivo studies. Grzesiak et al. used siRNA-transfection to knock down both the α2 and the β1-subunit in PDAC cells, which reduced proliferation, adhesion and migration when the cells were grown on a Col IV or Col I matrix. In vivo, β1-knocked PDAC cells injected into nude mice, produce smaller tumours and less metastasis compared to control-transfected cells (Grzesiak et al, 2011). Similarly, Sawai et al. demonstrated that several BM proteins, including Col IV, stimulate PDAC cell invasion in an integrin β1 dependent manner in vitro. These effect were associated with increased MAPK-signalling (Sawai et al, 2008).

Two integrin inhibitors have been evaluated in phase II trials including patients with metastatic PDAC. Inhibition of integrin αVβ3/αVβ5 (cilengitide) with the purpose of targeting endothelial cells had no beneficial effect compared to Gemcitabine alone (Friess et al, 2006). Volociximab, an integrin α5β1 inhibitor has been evaluated in a phase II trial, but the final results have not yet been published (clinicaltrials.gov, NCT00401570).

Chapter 16 – Biomarkers derived from the tumour stroma

16.1 Type IV collagen

Plasma Col IV levels were increased in PDAC patients before surgery and persistent high postoperative levels were associated with an unfavourable prognosis. A possible explanation for this association could be that surviving PDAC cells at the site of the primary tumour or in undetectable metastases continue to secrete Col IV. Alternatively, an increased activity of Col IV degrading enzymes MMP2- and/or -9 might release Col IV fragments into the circulation upon matrix invasion. The latter could also explain the survival association, as high MMP-2 expression has been associated with poor prognosis in PDAC (Zhai et al, 2015).

The BM expression of Col IV has been reported to be progressively lost with PDAC progression and switched to an increased expression in the tumour stroma (Lee et al, 1994; Linder et al, 2001). Those claims are not supported by the findings in this thesis. Here, most PDAC tumours expressed high levels of Col IV in both BM-like structures near cancer cells and in the stroma surrounding these clusters. Additionally, high tissue expression of Col IV in both these compartments trended towards an association with improved survival, although non-significant. One would expect an opposite trend considering the tumour promoting effects of Col IV in vitro and the association between plasma levels and survival. However, that assumes a causal relationship between tissue expression levels and tumour aggressiveness.

Likely, other aspects than mere Col IV staining have to be taken into account to understand its significance. As an example, Col I has been shown
to promote PDAC proliferation, invasion and survival *in vitro* (Armstrong et al., 2004; Grzesiak & Bouvet 2006; Grzesiak et al., 2011). On the contrary, high abundance of fibrillar collagens (i.e. Col I/III) in tumour tissue has been associated with longer survival (Erkan et al., 2008). But recently, Laklai et al. found that while the total abundance of fibrillar collagens did not predict outcome in their data, there was a significant association between the thickness of collagen fibers and poor survival (Laklai et al., 2016). Another recent study showed that PDAC patients with highly aligned collagen fibers in their tumours have an unfavourable prognosis (Drifka et al., 2016). Those findings suggest that the tissue architecture of stromal collagens are important in how they affect tumour behaviour and underscore the need of a more complex understanding of the ECM in PDAC.

Another possible explanation is that Col IV might exert different effects in the much more complex microenvironment *in vivo* compared to monolayer cell culture. Monolayer cell models inevitably fail to recapitulate interactions with other ECM components as well as putative tissue tension effects caused by collagen deposition and cross-linking between collagen molecules. Additionally, cell culture can theoretically select for populations that secrete Col IV in order to strive in artificial systems.

### 16.2 Endostatin

The anti-angiogenic Col XVIII fragment Endostatin was elevated in plasma samples before surgery, and was normalised postoperatively, confirming previous observations (Ohlund et al., 2008). Moreover, high pre- and postoperative plasma levels of Endostatin were associated with worse outcome. These results are in line with the observed anti-angiogenic effect of supernatant from PSC-PDAC cell co-culture in vitro, and with the negative prognostic impact associated with tissue hypoxia in PDAC tumours (Shibaji et al., 2003; Erkan et al., 2009). Assuming that the primary tumour is the principal source of Endostatin, the decreased levels in the postoperative setting could be due to surgical removal of that source. Another possible explanation might be a systemic postoperative decrease of Endostatin in order to improve angiogenesis in the healing surgical wound.

### 16.3 Matricellular proteins

Both TNC and OPN were overexpressed in PDAC tumour tissue compared to normal pancreatic tissue, and were increased in plasma samples from PDAC patients compared to healthy controls. Additionally, high postoperative levels of OPN were associated with worse prognosis, suggesting that plasma OPN is a potential prognostic marker in PDAC. A relation between OPN and tumour aggressiveness is supported by *in vitro* findings. Kolb et al. showed that OPN interact with CD44 to stimulate PDAC cell invasion (Kolb et al., 2005).
On the contrary, the results of the TMA stainings suggest that OPN is associated with tumour protective mechanisms. High expression of OPN in the stroma was an independent predictor of longer survival. These findings are supported by another study where tissue OPN expression was scored in PDAC cells, showing that patients with OPN positive tumours live longer (Collins et al, 2012).

OPN has been suggested to act tumour protective by affecting the immune system in other cancer forms. OPN null mice are more prone to develop tumours and display a weaker host immune response against tumours (Crawford et al, 1998; Hsieh et al, 2012). A recent study showed that OPN is important for natural killer T cell recruitment in a prostate cancer mouse model (Danzaki et al, 2016). Since macrophages have been shown to express OPN mRNA in PDAC, it’s possible that stromal OPN expression is a surrogate marker for macrophage infiltration (Sedivy et al, 2005). Considering the conflicting results on the role of OPN, more studies are needed to understand its role in the PDAC microenvironment.

16.4 Hyaluronan
Here, stromal HA expression did not predict postoperative survival, in contrast to previous reports (Cheng et al, 2013; Whatcott et al, 2015). Those studies however, either used an antibody with questionable specificity or used a scoring method that is profoundly affected by cellularity.

Preclinical studies have shown promising effects of stromal HA depletion (Jacobetz et al, 2012; Provenzano et al, 2012; Chauhan et al, 2013), and hyaluronidase treatment is tolerable in patients with metastatic PDAC (Hingorani et al, 2016). As presented in this thesis, stromal HA was strongly expressed in almost all tumours and lymph node metastases with much less variation between individual tumours compared to other stromal components. These findings suggest that HA deposition is a universal trait of the desmoplastic PDAC stroma. If HA depletion therapies prove to be beneficial in ongoing clinical trials, it will potentially have a considerable impact since it is a target expressed in both primary tumours and metastases in almost all PDAC patients.

Serum (S)-HA levels were increased in PDAC patients compared to healthy controls. This supposedly reflects a S-HA release from the tumour stroma in PDAC. ROC analysis yielded AUCs of 0.84, and the sensitivity and specificity for both assays were close to that of Ca 19-9 reported in larger patient cohorts (Poruk et al, 2013). However, the molecular mass of S-HA seems to influence its use as a diagnostic biomarker in elderly (aged ≥80 years). Age adjustments were feasible when the sandwich assay was used, but not when the competitive assay was used, which has a higher sensitivity for low molecular mass (M) HA. For some reason, low M HA does not seem to increase with age in patients with PDAC tumours. Future studies should
aim to map out the HA size profiles in PDAC and healthy individuals, both in the circulation and in the tumour tissue.

S-HA was correlated with serum bilirubin, indicating that bile duct obstruction may constitute a potential confounder in the interpretation. A possible explanation for this finding is that circulating HA is mainly degraded in sinusoidal endothelial cells in the liver (Fraser et al, 1985). The function of these cells is impaired when the bile duct is obstructed in mice, causing increased levels of HA in the circulation (Yoshidome et al, 2000).

Taken together, this suggests that the molecular mass of circulating HA influences its potential as a PDAC biomarker and become an increasingly important confounder to consider with increasing age. To fully elucidate the value of S-HA as a diagnostic and prognostic tool for PDAC management, future studies need to involve larger cohorts, including controls with benign causes of bile duct obstruction.

16.5 Combining stromal and cell derived biomarkers in PDAC
Circulating levels of the stromal components Col IV, Endostatin, OPN and TNC were measured and compared to conventional tumour markers that are expressed by cancer cells (Ca 19-9, Ca 125, CEA and TPS). At a fixed specificity of 95 %, the overall sensitivity for the stromal markers were lower than the conventional markers, and none outperformed Ca 19-9. However, stromal markers were up regulated in cases where conventional markers were not increased, which suggests that combinations of markers can increase the sensitivity.

High plasma levels of Endostatin, Col IV, OPN and Ca 125 were associated with poor survival. While studies involving larger patient cohorts have demonstrated that Ca 19-9 and CEA levels can predict PDAC outcome (Poruk et al, 2013; Liu et al, 2015), no such associations were found here. However, the small sample size increases the risk of bias. Additionally, variation in inter-individual levels was higher for the conventional markers, which affect significance testing. Alternatively, the predictive value of circulating Col IV, Endostatin, OPN and Ca 125 are stronger, and thus reach significance even in small sample studies.

Elevations of stroma-derived biomarkers, such as collagens and hyaluronan, are not specific for PDAC tumours, since they are readily expressed in various tissues under physiological conditions and diseases (Toole 2004; Shoulders & Raines 2009). On the other hand, the same is true for clinically used biomarkers including Ca 19-9, which increases in numerous non-malignant conditions (Ballehaninna & Chamberlain 2012). It is unlikely that a single biomarker will be discovered that can detect all PDAC patients with high specificity. But combinations of biomarkers could possibly increase the accuracy, by reflecting both cancer cell and stromal compartments. This was underscored by Chan et al., presenting a promising
biomarker combination including circulating Ca 19-9, Ca 125 and the basement membrane protein laminin gamma 2 (LAMC2). The combination outperformed Ca 19-9 alone in discriminating PDAC from healthy controls and benign pancreatic diseases, in three individual cohorts including over 400 samples (Chan et al, 2014).

Taken together, this suggests that while the stromal markers evaluated here overall have lower accuracies, they may be useful in combination with conventional, cell-derived, biomarkers. However, such combinations, including the markers evaluated in this thesis, need to be validated in larger patient cohorts in order to draw more rigorous conclusions regarding their potential clinical use. In light of the results of this thesis, a biomarker combination of HA, Col IV, Endostatin, OPN, Ca 19-9 and TPS seems promising to evaluate further.

16.6. CD44 receptors as tissue biomarkers and drug targets

Here, high expression of both CD44s and CD44v6 in PDAC tissue was associated with poor survival, confirming previous findings (Hong et al, 2009; Lee et al, 2014; Li et al, 2014; Li et al, 2015; Xiaoping et al, 2015). Additionally, high CD44s predicted poor outcome after adjusting for known predictive factors. This underscores an important role for CD44 receptors in the context of tumour promoting stroma interactions in PDAC. Since HA was found to be strongly expressed in almost all PDAC tumours, CD44 levels rather than ligand availability seems to regulate CD44-HA dependent cellular events.

Blocking CD44-ECM interactions reduces tumour relapse after oncological treatment in PDAC mouse models (Lee et al, 2014; Molejon et al, 2015). Hence, anti-CD44 antibodies are potential therapeutic alternatives in the adjuvant setting to prevent cancer recurrence. Trials targeting CD44 receptors have been conducted, including patients with metastatic cancer of various origins. Phase I trials have assessed the safety and preliminary efficacy of both bivatuzumab meransine, a CD44v6 antibody, and RG7356, an antibody that targets the HA interacting domain of all CD44 isoforms (Tijink et al, 2006; der Houven van Oordt et al, 2016). However, all bivatuzumab meransine (anti-CD44v6) trials were discontinued due to one case of lethal skin toxicity (Tijink et al, 2006). While RG7356 was proven to be tolerable in patients with metastatic solid tumours, the study was terminated due to a lack of clinical response (der Houven van Oordt et al, 2016). That particular study included 65 patients with 20 different tumour types, including patients with low CD44s expression in pre-treatment biopsies. Such poorly defined inclusion criteria makes efficacy interpretations uncertain. Most likely, the response to anti-CD44 treatment is different in different tumour types and also related to expression levels and the specific isoform profile expressed on the cancer cells. The latter is
supported by a study using xenograft models of various cancers. Tumour xenografts that predominantly express CD44s are more likely to respond to RG7356, compared to tumours that predominantly express CD44v (Birzele et al, 2015).

Chapter 17 - Plasma miRNAs as biomarkers in PDAC

It is crucial to find biomarkers that can detect PDAC prior to the symptom debut in order to improve the overall survival. Despite this, few studies have included prediagnostically collected samples in biomarker discovery, since cohorts such as the VIP cohort are rare globally. Mayers et al. profiled plasma metabolites and found that branched-chained amino acids (BCAA) are elevated in prediagnostic samples from PDAC patients, and that BCAA increase is associated with a 2-fold increased risk of developing PDAC (Mayers et al, 2014). Ca 19-9 increases over a year before the diagnosis date in prediagnostic samples, but has a low sensitivity and specificity for early PDAC detection (Nolen et al, 2014; O’Brien et al, 2015).

Several studies have demonstrated that circulating miRNAs are altered in stage I-II PDAC at diagnosis, resulting in high hopes for the use of miRNAs in early discovery of PDAC (Schultz et al, 2014; Srivastava et al, 2014; Xu et al, 2016). This thesis presents the first study that measure plasma miRNA levels in prediagnostic samples with the aim of early PDAC detection. However, the results suggest that miRNAs are not useful for early PDAC discovery. Similar to previous reports, Ca 19-9 was increased in prediagnostic samples, but had low sensitivity and specificity.

Fifteen plasma miRNAs were significantly altered in stage I-II PDAC patients at diagnosis compared to healthy controls. Most of these miRNAs have previously been reported as being altered in blood samples from PDAC patients, including increased let-7d, miR-22, -24, -34a, -122, -130b, -574 and -885, and decreased miR-106b and -144 (Bauer et al, 2012; Liu et al, 2012; Ganepola et al, 2014; Schultz et al, 2014; Ali et al, 2015; Xu et al, 2016). Novel findings include the increased levels of miR-197 and decreased levels of miR-101. A multivariate statistical model combining the candidate miRNAs outperformed Ca 19-9 in ROC analysis. However, neither individual candidate miRNA nor the combination model could separate PDAC patients from healthy controls prior to PDAC diagnosis. This suggests that the miRNA alterations found here appear late in the disease course, and the results are discouraging in terms of early detection applications for plasma-miRNAs.

miRNAs are altered in tissue from PanIN-lesions (Yu et al, 2012) and this should theoretically also cause altered plasma levels early in the disease progression. On the other hand, miRNA dysregulation in primary tumours
and in the circulation are poorly correlated in several cancer forms, including PDAC (Jarry et al, 2014; Kojima et al, 2015). The circulating miRNAs released from tumours will be diluted with miRNAs released from other tissues, which might explain the poor correlation. Alternatively, the tissue release of miRNAs into the circulation is a selective process. Supporting the latter is a study showing that abundant intracellular miRNAs in breast cancer cells are not released extracellularly in corresponding amounts (Pigati et al, 2010). This suggests that cancer cells preferentially release certain miRNAs and retain others and might explain the lack of early miRNA alterations in plasma.

In this thesis, the candidate miRNAs were discovered in patients diagnosed at stage I-II, but most patients in the prediagnostic cohort had stage III-IV PDAC at diagnosis. If PDAC progression from the initial mutational event to metastatic cancer takes over 15 years, as suggested by Yachida et al. (Yachida et al, 2010), then several patients in the prediagnostic cohort should have been at an early PDAC stage when the prediagnostic samples were collected. Importantly, the data presented by Yachida et al. is based on only seven patients, and the model relies on several uncertain assumptions. This includes the quantification of S-phase using Ki-67 stainings and estimates of cell doubling times and rates of mutational acquisition. An alternative hypothesis is that the progression from early stage PDAC to metastatic disease is more rapid than suggested by Yachida et al. This is supported by Yu et al., who compared the average adjusted age at diagnosis over TNM stages using a dataset including over 11,000 PDAC patients (Yu et al, 2015). They found that patients diagnosed with advanced disease are only 1.2 years older on average than patients diagnosed at stage I, suggesting a rapid progression from localized to metastatic disease. Moreover, the median survival in non-resected stage IA patients is only 6.8 months, compared to 24.1 months for resected stage IA PDAC, based on a large patient material (Bilimoria et al, 2007). Importantly, non-resected stage I patients likely constitute a selected population that is older and with significant co-morbidity. However, the extreme difference in median survival is also likely to reflect disease progression to some extent, as most PDAC patients die from metastatic disease.

Chapter 18 – Future directions

18.1. The role of stromal components in PDAC pathogenesis
PDAC cells secrete additional stromal components apart from Col IV, such as Col III, vitronectin and laminin (Lohr et al, 1994). These proteins might also affect PDAC progression via similar autocrine loops. This would be interesting to study using a similar approach as in paper I. Additionally, the
regulation of PDAC cell secretion of Col IV was not studied within the scope of this thesis nor where the effects of PSC interactions on Col IV synthesis. Similarly, downstream effects of Col IV-integrin interaction in PDAC cells remain to be elucidated.

This thesis demonstrates an association between high OPN expression and better prognosis in PDAC, but the reason for this is unknown. Analysis of co-localisation and interactions with immune cells using PLA in the TMA is a reasonable starting point for future studies.

Chauhan et al. reduced stromal HA and Col I to increase chemotherapy deliverance in a mouse model, by administering an angiotensin-II receptor (ATIIR) blocker that is commonly used as a hypertensive medication (Chauhan et al, 2013). However, it is unknown whether ATIIR blockers actually affect the stroma in human PDAC patients. The TMA generated in paper III can be used to determine whether the HA and collagen deposition differs in PDAC patients that were medicated with an ATIIR blocker before the diagnosis and whether an association with overall survival can be observed.

18.2. Biomarker discovery

Ca 19-9 is still the only clinically used PDAC biomarker despite its many drawbacks. Tissue biomarkers, such as hENT1 that can aid in postoperative treatment decisions have not been implemented in clinical decision-making. Why haven’t better biomarkers been discovered? And why doesn’t promising biomarkers reach clinical use? Basically, more rigorous validation is demanded now, compared to when Ca 19-9 was approved, which is reasonable. It is not enough to present promising data based on a single cohort. The findings have to be validated in external cohorts and include patients with differential diagnoses, such as benign pancreatic disease and other cancer forms.

This is hard to accomplish in a small centre, such as Umeå University Hospital. In order to access sufficient sample sizes for future studies and validation of the tissue and blood-based biomarkers presented here, the next step is thus to engage in collaborations. A collaboration has been initiated with Sahlgrenska University Hospital to expand the TMA cohort, apart from including more cases from Umeå University Hospital. The final TMA is expected to include over 400 PDAC cases, and will constitute one of the largest PDAC TMAs in the world.

Circulating miRNA profiles overlap between different types of cancers (Jarry et al, 2014). Similarly, the altered miRNAs found in paper IV are not specific for PDAC. Perhaps common plasma miRNA alterations occur secondary to general cancer associated hallmarks such as genomic instability and hyperproliferation? If so, the use of miRNAs in distinguishing between
cancer forms may be unfeasible. On the other hand, circulating miRNAs might be useful to detect common cancer-associated events.

Candidate miRNAs were discovered by screening for a large number of miRNAs in patients with manifest PDAC. However, it is possible that a different set of miRNAs can be associated with early disease progression, but normalise close to symptom presentation. Additionally, over 2500 miRNA species have been described, so there might exist miRNAs that increase in early PDAC that were simply not screened for here. Ideally, an unbiased miRNA screening should be carried out in prediagnostic samples from the VIP cohort and preferentially by including patients that develop different cancer forms. By such an approach, it would be possible to detect early miRNA changes involved with cancer risk in general, and differentiate miRNAs alterations associated with particular cancer forms.

18.3. Identification of risk groups
The low prevalence in the general population makes it difficult to develop effective screening programs for PDAC, as diagnostic biomarkers inevitably have low positive predictive values (PPV). As an example, a test with a sensitivity and specificity of 99.5% would have a PPV of 6.4% if the test was carried out in Swedes between 55-74 years of age (prevalence ~0.03%) (Socialstyrelsen 2015). This means that only 6.4% of the individuals classified as PDAC cases by the test would truly have the disease, and over 90% would be wrongly classified.

Thus, risk groups for PDAC must be identified in order to defend any screening program ethically and economically. Interestingly, almost 90% of patients with PDAC have impaired glucose tolerance or manifest diabetes at diagnosis, which is unique among solid tumours (Pannala et al, 2008; Aggarwal et al, 2013). While manifest diabetes carries a too low relative risk increase for effective screening (Ben et al, 2011), few studies have investigated the role of glucose intolerance as an early risk marker for PDAC. This can be studied reliably in the VIP cohort, as the participants undergo an oral glucose tolerance test (OGTT) as part of the routine health examination. A cohort study has been initiated, which will use VIP data to elucidate the risk for PDAC in individuals with impaired glucose tolerance.
CONCLUSIONS

• High CD44s and low stromal OPN expression in primary tumours predicts poor survival independently of well-established predictors of survival.

• HA expression is a universal trait of the PDAC stroma in primary tumours and metastases, but is not associated with patient survival.

• Col IV is expressed in BM-like structures that surround cancer cell clusters in most PDAC tumours and lymph node metastases. The expression in BM-like structures is paralleled with Col IV deposition in the surrounding stroma, and high expression in both compartments trend towards an association with longer median survival.

• PDAC cells grown in vitro synthesise Col IV, which stimulates proliferation, migration and apoptosis evasion via an autocrine loop. Proliferation depends on the CB3 domain on Col IV and the Col IV binding integrin subunit β1.

• PDAC patients present with elevated levels of circulating Col IV, Endostatin, TNC, OPN and HA before surgery and all except Endostatin remain elevated postoperatively.

• High preoperative plasma Endostatin as well as high postoperative plasma Endostatin, Col IV and OPN are associated with poor survival.

• Stroma-derived markers have lower sensitivities compared to Ca 19-9, Ca 125 CEA and TPS, but have stronger associations with survival.

• Serum-HA levels predict the postoperative survival independent of well-established prognostic factors.

• Several plasma miRNAs are altered in stage I-II PDAC patients, but since alterations appear late in the disease course, these miRNAs are not suitable biomarkers for early PDAC discovery.
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