PANCREATIC DUCTAL ADENOCARCINOMA
– THE ROLE OF TUMOR-ASSOCIATED
MACROPHAGES AND SYSTEMIC
INFLAMMATORY RESPONSE

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nee Koski

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki, for public examination in lecture room 2, Biomedicum 1, on 3 March 2017, at 12 noon.

Helsinki 2017
To My Family
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1 ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest forms of cancer. Options for treatment are limited, and the only possibility of cure is radical surgery combined with chemotherapy. Inflammation and tumor stroma are important mediators in PDAC progression. Tumor-associated macrophages (TAMs), among other cells, create an immunosuppressive microenvironment and enhance tumor progression. Because they pivotally participate in tumorogenesis, TAMs are a potential target for therapeutic intervention.

The aim of these studies was to explore inflammation and TAMs in PDAC. Three of the studies were conducted in cell cultures, and one was a retrospective clinical study. We polarized macrophages in cell cultures towards inflammatory M1 and anti-inflammatory M2 phenotypes and assessed the changes in the signaling pathways and the effect they had on pancreatic cancer cell migration. We studied the association of preoperative systemic inflammatory response (SIR), based on laboratory data, with the outcome of 265 patients with resectable PDAC.

Tumor-associated anti-inflammatory M2 macrophages promoted pancreatic cancer cell migration in co-cultures by activating their MMP9 and ADAM8 expression. Support of the pro-inflammatory M1 phenotype causes these macrophages to inhibit cancer cell migration. Several intracellular STAT pathways and the NFkB pathway were activated by the interactions of cancer cells and macrophages.

In preoperative laboratory data, patients’ elevated C-reactive protein (CRP), an indicator of SIR, predicted worse postoperative survival. Moreover, low levels of albumin, the most abundant protein in human blood circulation, as well as elevated tumor markers CA19-9 and CEA, were associated with worse survival.

These studies provide novel insight into the interaction of TAMs and PDAC. The results encourage further research into TAMs and exploration of the possibilities of skewing macrophage polarization toward the inflammatory M1 phenotype. Development of SIR seems detrimental for patients with PDAC and predicts worse outcome. Preoperative CRP, in combination with albumin and tumor markers and clinical data, could prove useful when evaluating patients’ prognosis.
Haimasyövällä on huono alle 8% viiden vuoden kokonaiseloonjäämisennuste ja se onkin Suomessa kolmanneksi yleisin syöpäkuoleman aiheuttaja. Tulehduksen ja siihen liittyvien solujen, kuten makrofagien, on todettu olevan oleellisena tekijänä haimasyövän invaasiossa. Kudoksissa verenkierrosta saapuvat monosyytit erilaistuvat niiden mikroympäristön vaikutuksesta joko inflammatorisiksi M1-tyyppin makrofageiksi, jotka aktivoivat tulehdsreittejä ja vastustavat syövän syntymistä ja etenemistä, tai anti-inflammatorisiksi M2 makrofageiksi, jotka puolestaan aktivoivat kudosten korjasmekanismeja ja muun muassa verisuonien muodostusta. Syöpäsolujen läheisyydessä monosyytit erilaistuvat tuumariassosioiduksi makrofageiksi, joiden on todettu lisäävän haimasyöpäsolujen invaasiota ja jakautumista, sekä olevan merkki huonosta ennusteesta. Tutkimuksen tarkoituksena oli tarkastella eri tavoin erilaistettujen makrofagien ja haiman duktaalisen adenokarsinooman välisiä vuorovaikutuksia. Lisäksi tutkimme ennen haimasyöpäleikkausta otettujen peruslaboratoriokokeiden, erityisesti tulehdusmerkkiaineiden, ennusteellista merkitystä haiman duktaalisessa adenokarsinoomassa.


Ennen haimasyöpäleikkausta otettuja verinäytteitä analysoimalla kävi ilmi, että haimasyöpäpotilaiden yleistynyt tulehdsreaktio, joka voidaan todeta muun muassa C-reaktiivisen proteiinin (CRP) kohonneena pitoisuutena, ennustaa lyhempää leikkaushen jälkeistä elinkaikaa. Lisäksi matala albumiinitaso sekä kohonnut haimasyövän merkkiaineet (CA19-9 ja CEA) olivat yhteydessä huonompaan ennusteeseen. Tulehdus- ja syöpämerkkiaineet saattaisivat siis olla avuksi potilaiden ennusteen arvioinnissa ennen haimasyöpäleikkausta. Tutkimuksemme kannustaa jatkamaan tulehduksen ja haimasyövän välisen yhteyksien selvittämistä ja etsimään makrofagien erilaistemiseen vaikuttamisen kautta mahdollisia kohteita lääkehoidoille.
3 LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which in the text are referred to by their Roman numerals (I-IV). The articles are reprinted with the kind permission of their copyright holders.


II. Increasing the Inflammatory Competence of Macrophages with IL-6 or with Combination of IL-4 and LPS Restrains the Invasiveness of Pancreatic Cancer Cells. Salmiheimo ANE, Mustonen HK, Vainionpää SAA, Shen Z, Kemppainen EAJ, Seppänen HE, Puolakkainen PA. J Cancer 2016; 7(1):42-49.


## 4 ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and a metalloproteinase</td>
</tr>
<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
</tr>
<tr>
<td>BRCA</td>
<td>Breast cancer susceptibility protein</td>
</tr>
<tr>
<td>CA</td>
<td>Coeliac axis</td>
</tr>
<tr>
<td>CA19-9</td>
<td>Carbohydrate antigen 19-9</td>
</tr>
<tr>
<td>CCL</td>
<td>C-C chemokine ligand</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>Cyclin-dependent kinase inhibitor 2A</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSF-1</td>
<td>Colony-stimulating factor-1</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra cellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ERCP</td>
<td>Endoscopic retrograde cholangiopancreaticography</td>
</tr>
<tr>
<td>EUS</td>
<td>Endoscopic ultrasound</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FOLFIRINOX</td>
<td>Folinic acid, fluorouracil, irinotecan, and oxaliplatin</td>
</tr>
<tr>
<td>GA</td>
<td>Gastroduodenal artery</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GPS</td>
<td>Glasgow prognostic score</td>
</tr>
<tr>
<td>HA</td>
<td>Hepatic artery</td>
</tr>
<tr>
<td>hENT1</td>
<td>Human equilibrative nucleoside transporter 1</td>
</tr>
<tr>
<td>HGF-Met</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HR</td>
<td>Hazard ratio</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>Insulin-like growth factor binding protein-1</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>IPMN</td>
<td>Intraductal papillary mucinous neoplasm</td>
</tr>
<tr>
<td>ISGPS</td>
<td>International Study Group of Pancreatic Surgery</td>
</tr>
<tr>
<td>IVC</td>
<td>Inferior vena cava</td>
</tr>
<tr>
<td>LNR</td>
<td>Lymph node ratio</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M1</td>
<td>Type 1 macrophage (inflammatory)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>M2</td>
<td>Type 2 macrophage (anti-inflammatory)</td>
</tr>
<tr>
<td>MCN</td>
<td>Mucinous cystic neoplasm</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>mGPS</td>
<td>Modified Glasgow prognostic score</td>
</tr>
<tr>
<td>Mic-1</td>
<td>Macrophage inhibitory cytokine-1</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MRCP</td>
<td>Magnetic resonance cholangiopancreaticography</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>No/1</td>
<td>Lymph-node status</td>
</tr>
<tr>
<td>NCCN</td>
<td>National Comprehensive Cancer Network</td>
</tr>
<tr>
<td>NLR</td>
<td>Neutrophil to lymphocyte ratio</td>
</tr>
<tr>
<td>NT siRNA</td>
<td>Non-targeting siRNA</td>
</tr>
<tr>
<td>P6</td>
<td>Pyridone 6</td>
</tr>
<tr>
<td>PALB2</td>
<td>Partner and localizer of BRCA2</td>
</tr>
<tr>
<td>PanIN</td>
<td>Pancreatic intraepithelial neoplasia</td>
</tr>
<tr>
<td>PDAC</td>
<td>Pancreatic ductal adenocarcinoma</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PLR</td>
<td>Platelet to lymphocyte ratio</td>
</tr>
<tr>
<td>PV</td>
<td>Portal vein</td>
</tr>
<tr>
<td>R0/1</td>
<td>Resection margin status</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RR</td>
<td>Risk ratio</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SFM</td>
<td>Serum free media</td>
</tr>
<tr>
<td>SIR</td>
<td>Systemic inflammatory response</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SMA</td>
<td>Superior mesenteric artery</td>
</tr>
<tr>
<td>SMV</td>
<td>Superior mesenteric vein</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted protein acidic and rich in cysteine</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumor-associated macrophage</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of matrix metalloproteinase</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumor node metastasis</td>
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<tr>
<td>TP53</td>
<td>Tumor protein p53</td>
</tr>
<tr>
<td>TPA</td>
<td>Tissue polypeptide antigen</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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</table>
Pancreatic cancer is notorious for its dismal prognosis, with an overall 5-year survival rate of less than 8% (Siegel 2016). Although by incidence it is in Finland only the tenth most common cancer in men and ninth in women, it is still the third most common cause of cancer deaths in both sexes (Finnish Cancer Registry). Pancreatic ductal adenocarcinoma (PDAC), accounting for over 90% of pancreatic cancers, metastasizes aggressively in its early stages. Other tumors of the pancreas have different prognoses and treatment strategies (Fischer 2014, Rezaee 2016), but the focus of this study is on PDAC.

The fundamental causes of pancreatic cancer remain unclear. It usually is a consequence of accumulating gene mutations (Vincent 2011, Ryan 2014). Some of the eminent risk factors include smoking and chronic pancreatitis. In the development and progression of pancreatic cancer, important components emerging in the past two decades have been inflammation and the cells associated with it, as well as stromal elements in the tumor microenvironment (Coussens 2002, Elinav 2013, Atsumi 2014). Its prognosis is based on stage of the disease, tumor-free resection margins, histological type and differentiation of the tumor, lymph-node metastases, and tumor size.

Radical surgical resection accompanied by oncological treatment remain the only chance for curative treatment; thus, early detection is crucial for the patient’s outcome. The central anatomical position of the pancreas enables cancer to spread locally to adjacent structures such as the duodenum, great vessels, common bile duct, and peritoneum. Further, pancreatic cancer has a high tendency to spread to lymph nodes and through the bloodstream to distant organs (Ryan 2014).

The aim of this study was to examine the association between pancreatic cancer and inflammation, and especially tumor-associated macrophages.
6 REVIEW OF THE LITERATURE

6.1 Anatomy and function of the pancreas

The pancreas lies retroperitoneally in the upper, central abdomen. Its central position adjacent to large vessels such as the coeliac and superior mesenteric arteries (which are partially responsible for the blood supply to the pancreas), the abdominal aorta, inferior vena cava, and the splenic and superior mesenteric veins, both draining into the portal vein, makes the pancreas a challenging surgical target (Figure 1). Anatomically, the pancreas is divided into the head, body, tail, and the uncinated process. The main pancreatic duct (duct of Wirsung) joins the common bile duct in the head of the pancreas and leads the pancreatic and bile juices into the duodenum. The pancreatic tail is adjacent to the spleen. Both sympathetic and parasympathetic nervous systems innervate the pancreas (Textbook of Gastroenterology 2003).

The pancreas plays a critical role in food digestion and regulation of blood glucose levels. Functionally it is divided into the exocrine and endocrine pancreas. The exocrine pancreas (85% of all pancreatic tissue) synthesizes and secretes digestive enzymes such as amylase, trypsin, and lipase, into the duodenum. Pancreatic hormones (such as insulin, glucagon, somatostatin, amylin, and pancreatic polypeptide), secreted into the bloodstream by the endocrine pancreas (comprising the islets of Langerhans), are among their other functions necessary for glucose metabolism (Textbook of Gastroenterology 2003).
Figure 1. Anatomy and surgical resectability of pancreatic cancer. Figure reproduced with permission from Ryan DP, Hong TS & Bardeesy N 2014, "Pancreatic adenocarcinoma", New England Journal of Medicine, vol. 371, no. 11, pp. 1039-1049. Copyright Massachusetts Medical Society.
6.2 Epidemiology and survival of pancreatic cancer

Pancreatic cancer is the world’s 12th most common cancer type with approximately 340,000 new cases annually. It is nevertheless the seventh leading cause of cancer deaths worldwide with annual incidence and mortality rates being close to equal (Ferlay 2014). Of the 46 cancer types studied in the vast European population-based EUROCASE-5 study, pancreatic cancer had the worst age-standardized 5-year relative survival of 6.7% (De Angelis 2014) (Figure 2). In developed countries, pancreatic cancer incidence in 2012 was 8.6 in men and 5.9 in women per 100,000, with devastating mortality rates of 8.3 and 5.5. In less-developed areas, the incidence rate is 3.3 and 2.4 with equally grim mortality rates (Torre 2015).

![Figure 2. Stage distribution and 5-year relative survival rate of pancreatic cancer patients by stage at diagnosis. Stage distribution do not sum to 100% due to incomplete information. The percentages are according to Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin. 2016 Jan-Feb;66(1):7-30.](image-url)
According to the Finnish Cancer Registry, in Finland, the pancreatic cancer age-adjusted incidence rate in 2014 was 8.9 for men and 7.6 for women per 100,000 being responsible for 3.4 to 3.8% of total cancer incidence (Table 1). It is the third leading cause of cancer deaths in both men (after lung and prostate cancers) and women (after breast and lung cancers), with mortality rates of 8.9 and 6.5 (Finnish Cancer Registry 2015). Although still grim, the pancreatic cancer prognosis has fortunately slowly improved; in Finland, the age-standardized overall 5-year survival rose from 3% (1999-2001) to 6% (2005-2007) (p=0.002) (Lepage 2015). At Helsinki University Hospital for those patients resected for PDAC between 2000 and 2013, the 5-year survival rate was 22% and at 10 years 14% (Seppänen 2016). PDAC with distant metastases remains incurable, with a 5-year survival rate near zero (Siegel 2013).

Table 1. The mean annual incidence and mortality of pancreatic cancer by time period. Source: Finnish Cancer Registry, Cancer Statistics at www.cancerregistry.fi, updated 5 March, 2016

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<td>Male</td>
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<tr>
<td>Incidence</td>
<td>229</td>
<td>264</td>
<td>275</td>
<td>315</td>
<td>308</td>
<td>337</td>
<td>366</td>
<td>456</td>
<td>520</td>
<td>536</td>
<td>531</td>
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<tr>
<td>Incidence rate*</td>
<td>9.9</td>
<td>10.5</td>
<td>9.9</td>
<td>10.5</td>
<td>9.4</td>
<td>9.3</td>
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<tr>
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<td>216</td>
<td>249</td>
<td>256</td>
<td>293</td>
<td>292</td>
<td>309</td>
<td>344</td>
<td>415</td>
<td>474</td>
<td>503</td>
<td>538</td>
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<tr>
<td>Mortality rate*</td>
<td>9.3</td>
<td>9.9</td>
<td>9.3</td>
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<td>8.9</td>
<td>8.5</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incidence</td>
<td>207</td>
<td>255</td>
<td>316</td>
<td>356</td>
<td>398</td>
<td>405</td>
<td>424</td>
<td>512</td>
<td>553</td>
<td>575</td>
<td>601</td>
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<tr>
<td>Incidence rate*</td>
<td>5.8</td>
<td>6.3</td>
<td>6.7</td>
<td>6.8</td>
<td>7.0</td>
<td>6.7</td>
<td>6.6</td>
<td>7.4</td>
<td>7.4</td>
<td>7.5</td>
<td>7.6</td>
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<tr>
<td>Mortality</td>
<td>195</td>
<td>241</td>
<td>302</td>
<td>331</td>
<td>377</td>
<td>383</td>
<td>393</td>
<td>478</td>
<td>525</td>
<td>523</td>
<td>545</td>
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<tr>
<td>Mortality rate*</td>
<td>5.4</td>
<td>5.9</td>
<td>6.4</td>
<td>6.2</td>
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<td>6.2</td>
<td>5.9</td>
<td>6.7</td>
<td>6.6</td>
<td>6.6</td>
<td>6.5</td>
</tr>
</tbody>
</table>

*Age-adjusted (to world standard population) rate per 100,000
6.3 Etiology

6.3.1 Risk factors

The causes of pancreatic cancer are multifactorial and complex, with precursor lesions often preceding the cancer. Lifetime risk for pancreatic cancer is approximately 1% in the developed world (Torre 2015). Advanced age, smoking, chronic pancreatitis, and a family history of pancreatic cancer are consistently associated with increased risk for pancreatic cancer. Other, less conclusive risk factors include diabetes mellitus, alcohol consumption, obesity, chronic cirrhosis, western dietary habits, and various occupational exposures. More recently, non-O blood group has also been associated with increased risk for pancreatic cancer (Hassan 2007, Hidalgo 2010, Vincent 2011).

5.3.1.1 Age

Risk for pancreatic cancer increases with age, being extremely rare in patients under age 40. The median age of onset is 65 to 75 years, with over 80% of patients diagnosed between 60 and 80 (Ghadirian 2003, Lowenfels 2006, Raimondi 2007). Smoking and genetic disorders seem to be more prominent risk factors for early-onset pancreatic cancer in younger than in older groups (Raimondi 2007).

5.3.1.2 Genetic predisposition

Approximately 10% of pancreatic cancer patients have a family history of the disease (Hruban 2010, Rustgi 2014). Even with only one first-degree relative affected, risk for pancreatic cancer is approximately twofold, and is as many as 57 times higher in families with four or more affected members than in unaffected families (Tersmette 2001, Klein 2004, McWilliams 2005). Of patients with familial pancreatic cancer, a germline BRCA2 gene mutation has been identified in 6 to 17% (Murphy 2002, Couch 2007) and germline PALB2 mutations in up to 3% (Jones 2009, Slater 2010). Some of the known hereditary cancer syndromes with an increased risk for pancreatic cancer are in Table 2.
Pancreatic cancer incidence is also associated with ethnic origin. In the 1980's, the highest incidence rates were reported in New Zealand Maoris, native Hawaiians, and African-Americans (Boyle 1989). In a more recent overview including 54 countries, the highest mortality rates for pancreatic cancer in 2007 were among men in the Czech Republic, Hungary, Slovakia, and the Nordic countries (Bosetti 2012). In the USA, risk for pancreatic cancer is considerably higher in the African-American population than in Caucasians (Olson 2013, National Cancer Institute SEER 2015).

Furthermore, pancreatic cancer risk associates with ABO blood-types but the mechanism is still undefined. Cohort studies indicate that patients with A, B, and AB blood types have a significantly higher risk for pancreatic cancer than patients with blood-type O (Egawa 2013, Wolpin 2009), and a genome-wide association study reported a similar association (Amundadottir 2009). In resectable pancreatic cancer, blood-type O may even have a favorable impact on prognosis (Ben 2011, Rahbari 2012).

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Affected Genes</th>
<th>Fold Increase in Relative Risk</th>
</tr>
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<tbody>
<tr>
<td>Hereditary breast and ovarian cancer</td>
<td>BRCA 1 and 2</td>
<td>6-10</td>
</tr>
<tr>
<td>Peutz-Jeghers</td>
<td>STK11/LKB1</td>
<td>132</td>
</tr>
<tr>
<td>Hereditary nonpolyposis colorectal cancer</td>
<td>hMLH1, HMLH2, others</td>
<td>8-9</td>
</tr>
<tr>
<td>Hereditary pancreatitis</td>
<td>PRSS1, PRSS2, SPINK1, CTRC</td>
<td>26-60</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>CFTR</td>
<td>5-6</td>
</tr>
<tr>
<td>Familial atypical multiple mole melanoma</td>
<td>CDKN2A</td>
<td>13-22</td>
</tr>
<tr>
<td>Familial adenomatous polyposis</td>
<td>APC</td>
<td>4-6</td>
</tr>
</tbody>
</table>

5.3.1.3 Chronic pancreatitis

Pancreatitis is an inflammation of the pancreas that can be divided into acute and chronic types. It can result for example from alcohol abuse, gallstones, and hereditary syndromes. Pancreatitis can also be an early manifestation or a misdiagnosis of pancreatic cancer. Independent of the underlying cause, there is, nonetheless, evidence of chronic pancreatitis as increasing risk for pancreatic cancer (Lowenfels 1993, Whitcomb 2002, Raimondi 2010), although only 4 to 6% of patients with chronic pancreatitis develop PDAC (Bang 2014). Typically PDAC appears about 10 to 20 years after the onset of chronic pancreatitis. The mechanism underlying the association between the diseases remains undetermined, and presumably comprises numerous steps with increasing DNA damage. For instance, KRAS mutations in healthy pancreatic tissue are very rare, but are common in chronic pancreatitis, and some suggest that KRAS mutations may, with time, lead to the development of pre-cancerous lesions (Whitcomb 2002, Raimondi 2010). Although chronic inflammation of the pancreas raises the risk for pancreatic cancer, no definite evidence exists that elevated circulating C-reactive protein (CRP) level, a common indicator of systemic inflammation, is associated with increased risk for PDAC (Douglas 2011, Bao 2013).

5.3.1.4 Diabetes mellitus

Diabetes mellitus (DM) is a metabolic disorder of the endocrine pancreas that, if left untreated, leads to elevated blood glucose levels. It is caused by either insulin deficiency (type 1 DM) or insulin resistance (type 2 DM). Type 2 DM is typically associated with overweight and the unhealthy nutritional habits, now with a rapidly rising incidence. Approximately 50 to 67% of pancreatic cancer patients have DM (Chari 2005), and studies have associated DM (primarily type 2) with an increased risk for pancreatic cancer (Everhart 1995, Giovannucci 2010, Batabyal 2014). The risk is greatest for a recently diagnosed DM, with risk declining over time, thus leading to the hypothesis that DM is a consequence, or an early indicator, of pancreatic cancer. Increased risk for pancreatic cancer has, however, also occurred in patients with an over-10-year history of DM, indicating that DM may also play a causal role in pancreatic carcinogenesis, possibly due to hyperinsulinemia, hyperglycemia, and inflammation (Giovannucci 2010, Li 2011, Bosetti 2014). Especially late-onset (>50 years of age) DM is associated with increased risk for pancreatic cancer, although only less than 1% of these subjects actually do develop pancreatic cancer. Of the pancreatic cancer patients, 20 to 25% have developed new-onset DM within 36 months prior to the cancer diagnosis (Chari 2005).
5.3.1.5 Lifestyle-related risks factors

The lifestyle factor showing the most consistent link with increased risk for pancreatic cancer is smoking. It is also the only environmental factor with evidence of a causative role, accounting for some 15 to 20% of the disease (Hassan 2007, Iodice 2008, Lynch 2009). Smokers have about a 75% increase in risk for developing pancreatic cancer as compared to never-smokers; the risk falls to non-smoker levels within approximately 10 to 15 years after quitting (Iodice 2008, Lynch 2009). Evidence of passive smoking and smokeless tobacco use as a cause is inconsistent (Duell 2012).

Obesity has become an increasing problem for the Western world. It is associated with increased risk for pancreatic cancer, among several other cancer forms, possibly by elevating insulin resistance and by causing inflammation, even without the formation of diabetes mellitus (Gukovsky 2013, Preziosi 2014). Besides obesity, low physical activity and dietary factors such as high intake of red meat, a high-fat, high-cholesterol diet, and low intake of marine omega-3-poly-unsaturated fatty acids, fruits, and vegetables may contribute to an increased risk (Ghadirian 2003, Raimondi 2009, Barone 2016). The role of vitamin D in risk for pancreatic cancer is controversial. Although epidemiological studies show vitamin D deficiency and low exposure to ambient ultraviolet radiation (sunlight) to be associated with increased risk, dietary intake of vitamin D has shown no association or even an adverse effect on risk for pancreatic cancer (Barreto 2015, Barone 2016).

The verification of heavy alcohol consumption as independently leading to pancreatic cancer formation has been inconclusive. Evidence from a pooled meta-analysis suggests that chronic heavy alcohol consumption (>30–40 g alcohol/day) probably elevates risk (Duell 2012). Further, various occupational exposures to such substances as metals, chlorinated hydrocarbon solvents, and ionizing radiation may increase risk (Ojajarvi 2000, Alguacil 2003, Santibanez 2010). In contrast to what was initially suspected, neither coffee nor caffeine is associated with increased risk for pancreatic cancer (Dong 2011, Turati 2012, Bhoo-Pathy 2013).
6.3.2 Prevention and screening

Since pancreatic cancer is a significant cause of death worldwide, and options for treatment are limited, prevention and early detection are key factors in its management. Unfortunately, as the causes are complex, multifactorial, and in part unknown, prevention is complicated. Crucial to the prevention is tackling the major lifestyle risks: smoking, diabetes, obesity, excessive alcohol consumption, and alcohol-related chronic pancreatitis. Further, the high prevalence of these risk factors presents an obstacle to screening (Vincent 2011).

When treating DM, it seems that metformin (but not insulin) may reduce the risk for pancreatic cancer (Giovannucci 2010, Soranna 2012, Bosetti 2014). The current consensus recommends, however, that cancer risk should not affect one’s choice of diabetes therapy, at least unless the patient is at very high risk for cancer (Giovannucci 2010). Associations between inflammation and cancer have led to investigation of anti-inflammatory drugs in cancer prevention, but with inconsistent findings concerning effects of acetosalicylic acid (aspirin) in lowering pancreatic cancer risk (Larsson 2006, Tan 2011, Cui 2014, Yue 2014).

Screening for pancreatic cancer is problematic. As pancreatic cancer metastases eagerly, and its prognosis is dismal in the advanced stages, pressure is on to find early precursor lesions. This leads, though, to a risk of overtreatment. Further, since pancreatic cancer incidence is relatively low, and no simple and accurate screening method exists, we have no capacity to screen all individuals at slightly increased risk. Therefore, researchers strive to assess the best screening protocol for high-risk populations, such as those with a genetic predisposition or patients over age 50 with new-onset diabetes and a smoking history. In several studies, the most favorable screening method seems to be magnetic resonance imaging (MRI) or magnetic resonance cholangiopancreaticography (MRCP) (Bruenderman 2015, Del Chiaro 2015).
6.4 Pathogenesis

6.4.1 Precursor lesions

Preceding the development of invasive pancreatic cancer emerge progressive, non-invasive precursor lesions (Koorstra 2008a). The most common are pancreatic intraepithelial neoplasias (PanINs) (Koorstra 2008b). PanIN lesions are atypical, non-invasive, microscopic (<5 mm) duct lesions classified into three grades according to the level of their epithelial atypia, acquiring cumulative genetic alterations as they progress. The higher the PanIN classification grade, the closer the lesion is to evolving into invasive carcinoma. The pancreas, with increasing age, often contains low-grade PanIN-1 lesions, most of which never develop into invasive carcinomas. Molecular biomarkers and imaging are under investigation to detect pre-malignant PanIN lesions, but although some promising markers (such as non-coding microRNAs) have been detectable, they have not yet been adapted for clinical use (Haugk 2010).

Intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs) are cystic tumors of the pancreas that can also evolve into PDAC (Dudeja 2015). In contrast to PanINs, they are macroscopic, and may be detectable by radiological imaging techniques such as computed tomography (CT). They form a spectrum of cystic lesions from mild atypia (adenoma), moderate dysplasia (borderline), high-grade dysplasia (carcinoma in situ), to invasive carcinoma (Haugk 2010). Non-invasive IPMNs and MCNs have an excellent prognosis; surgical intervention is considered curative. National and international guidelines direct the follow-up and treatment of the cystic neoplasms of the pancreas (Tanaka 2012, Del Chiaro 2013).
6.4.2 Accumulation of gene mutations

The molecular biology of pancreatic cancer continues to offer challenges due to the complexity and heterogeneity of its genetic basis. Accumulation of successive gene mutations seems to be the preliminary step for PDAC development. For example, one genetic analysis of 456 PDAC specimens revealed 32 recurrently mutated genes that could be organized into ten main tumorigenic pathways (Bailey 2016).

Almost all fully established PDACs contain at least one of the four most common genetic alterations. Over 90% include a mutation of the oncogene KRAS, which leads to increased proliferation, cell survival, and suppressed apoptosis (Almoguera 1988, Hruban 1993, Ferro 2014). KRAS mutations are common also in early precursor lesions. Likewise, 80 to 95% of pancreatic malignancies carry an inactivation of the CDKN2A tumor suppressor gene, leading to the loss of cell-cycle regulator protein p16, followed by an increase in cell proliferation (Caldas 1994, Schutte 1997). Inactivation of CDKN2A typically occurs later in the evolution of precursor lesions than does abnormal KRAS activation, with increasingly higher frequencies according to the progressing PanIN grades (Gnoni 2013). Other frequently mutated tumor-suppressor genes found in pancreatic malignancies include TP53 (inactivated in 50-75% of the cases), a gene critical in apoptotic signaling and control of DNA damage during the cell cycle (Redston 1994). The fourth most commonly mutated gene is SMAD4 (inactivated in about 55% of the cases), an important regulator of the transforming growth factor-β (TGF-β) signaling pathway (Wilentz 2000). Considerably rarer mutations include BRCA2 and STK11 and other defects associated with hereditary cancer syndromes (Table 2).
6.4.3 Inflammation and stromal elements

In the early 1990’s, studies revealed that chronic inflammation, such as in chronic pancreatitis, amplifies the risk for cancer (Ekbom 1993, Lowenfels 1993, Coussens 2002, Whitcomb 2002, Raimondi 2010). Activation of the immune system occurs in response to injury when pancreatic acinar cells release inflammatory signals such as interleukin (IL) 6, IL-1b, and tumor necrosis factor (TNF) α. Initially, activated immune cells eliminate those pancreatic cells that have genetic alterations, but over time, the anticancer response becomes insufficient. In the process, pancreatic stellate cells start producing large amounts of ECM compounds, and more inflammatory cells invade. Slowly, a tumor is established, and its microenvironment, abundant with tumor-associated macrophages (TAMs), neutrophils, and CD (cluster of differentiation) 4+ T-cells, is highly immunosuppressive, allowing the tumor to grow and begin invasion (Neesse 2015). Furthermore, tumors themselves cause inflammation and can instigate a cancer-related systemic inflammatory response (SIR) (Atsumi 2014). Cancer-related SIR may be a mediator of some cancer-associated symptoms such as weight-loss, fatigue, pain, fever, and depression, ones resulting from circulating inflammatory cytokines such as IL-6 and TNFα (Bower 2013, Laird 2013).

Pancreatic cancer is characteristically hypovascular and is extremely stroma-rich, being composed of almost 90% extracellular matrix (ECM), with a complex assembly of fibroblasts, immune and neural cells, pancreatic stellate cells, blood vessels, and a vast collection of growth factors, adhesion molecules, and structural compounds such as collagens, fibronectin, and hyaluronic acid (Feig 2012) (Figure 3). Cancer cells and tumor-stroma cells promote secretion of the structural compounds of the ECM by activating multiple signaling pathways such as TGF-β, fibroblast growth factor (FGF), insulin-like growth factor 1 (IGF-1), hepatocyte growth factor (HGF-Met), and epidermal growth factor (EGF) (Neesse 2011). The tumor ECM is continuously remodeled by proteinases such as ADAMs (a disintegrin and a metalloproteinase) and MMPs (matrix metalloproteinase), these counteracted by tissue inhibitors of metalloproteinases (TIMPs), among others, all of which are important mediators in tumor-cell invasion. Members of the ADAM, MMP, and TIMP families are upregulated in pancreatic cancer and have been implicated in the invasion-metastasis cascade (Lunardi 2014, Sahin 2016).
Figure 3. Components of Pancreatic Cancer. Pancreatic cancer comprises several interactive elements such as pancreatic-cancer cells, cancer stem cells, and the dense tumor stroma. This complex system involves multiple activated pathways as well as numerous secreted cytokines and growth factors. Figure reproduced with permission from Hidalgo, M. 2010, "Pancreatic cancer", New England Journal of Medicine, vol. 362, no. 17, pp. 1605-1617. Copyright Massachusetts Medical Society.
Although genetically altered cancerous pancreatic cells are the preliminary step in tumor development, the cancer stroma participates actively in many tumor-sustaining actions (Figure 4). Cancer cells are able to recruit normal cells to serve the cancer cells’ own advantage (Hanahan 2012). Pancreatic cancers contain immunosuppressive infiltrating immune cells even in the absence of an inflammatory immune response, and these cells are present also in the early PanIN lesions. Oncogenic activation of KRAS in pancreatic cells leads to the transcription of granulocyte-macrophage colony-stimulating factor (GM-CSF) that promotes the recruitment of myeloid progenitor cells (Neesse 2015). Mutations of KRAS and inflammation also promote the recruitment of infiltrating CD4+ T lymphocytes, which, in turn, participate in PanIN formation by blocking the CD8+ T cell-mediated anticancer immune responses (McAllister 2014).

In response to the signaling by cancer cells and infiltrating immune cells, cancer-associated fibroblasts start abundantly secreting the structural compounds of the tumor stroma, probably reflecting distorted tissue-repair mechanisms (Hanahan 2012). In the tumor microenvironment, cancer-associated fibroblasts also play an immunosuppressive role by their interaction with T lymphocytes (Feig 2013). The stromal cells are active producers of, for example, pro-angiogenic and tumorigenic growth factors, they participate in the epithelial-to-mesenchymal transition of cancer cells, and thus participate in initiation of the invasion-metastasis cascade (Hanahan 2012, Chaffer 2011).

Pancreatic stellate cells are pancreas-specific myofibroblasts that functionally and morphologically resemble hepatic stellate cells. Abundant in chronic pancreatitis, they are the main producers of the ECM in PDAC (Bachem 1998). In surgically treated PDAC, high activity of pancreatic stellate cells correlates with worse prognosis (Erkan 2008).

The excessive stroma of PDAC functions also as a biophysical barrier to drug delivery and therefore is a mediator of PDAC’s characteristically high resistance to cancer chemotherapy. The continuous production of ECM raises the interstitial pressure, which compresses the capillaries and hinders blood perfusion, leading to hypoxia and decreased accessibility by anti-tumor agents (Heinemann 2014). The central role of the tumor microenvironment makes it a relevant focus for novel therapy targets. Possible therapies could prove effective not only by directly targeting the stromal cells but also by breaking down the structural compounds of the ECM, by relieving vessel compression (thus increasing drug delivery into the tumor), and by enhancing drug accumulation within the tumor (Neesse 2013).
Figure 4. Contributions of some activated stromal cells to progression of cancer. The figure illustrates the central roles of infiltrating immune cells, cancer-associated fibroblasts, and vascular cells in the hallmarks of cancer, necessary for tumor development and advancement. Figure reprinted from Cancer Cell, Vol 21, Douglas Hanahan, Lisa M. Coussens, Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment, Pages 309-322, Copyright 2012, with permission from Elsevier.
6.5 Diagnosis

6.5.1 Clinical presentation

The symptoms of pancreatic cancer depend on the location and the stage of the disease but, unfortunately, often develop only at late stages. Especially in the early course of the disease, symptoms are often vague and non-specific, such as weight loss, upper abdominal discomfort, and nausea. Tumors that develop in the head of the pancreas (60-70% of the cases) can cause obstructive cholestasis, indicated by painless jaundice that can lead to early disease-detection. Abdominal pain is more common in later stages, as well as back pain that appears when the cancer infiltrates the retroperitoneal space. Type II diabetes mellitus (present in at least half the patients) and malabsorption can also be manifestations reflecting functional aberrations of the endocrine and exocrine pancreas. Pancreatic tumors can obstruct the pancreatic duct, which may lead to pancreatitis. Less frequently, even duodenal obstruction and gastrointestinal bleeding can occur. Advanced stages often display systemic manifestations such as cachexia, venous thrombosis, and depression (Hidalgo 2010, Ryan 2014).

Clinical examination may reveal upper-abdominal resistance, jaundice (typically first detectable in the sclera), lymphadenopathy, hepatomegaly, painlessly enlarged gallbladder, and ascites. Distant metastases develop primarily in the liver, abdomen, and lungs. Abnormalities in routine blood tests may include hyperglycemia, anemia, hypoalbuminemia, abnormalities in liver-function tests, and variations in inflammatory biomarkers (Jamieson 2005, Hidalgo 2010).

6.5.2 Imaging

Often most practical for the initial examination of diffuse upper abdominal symptoms or suspected pancreatic tumors is abdominal ultrasound, since it is safe, readily available, and cost-effective, but its sensitivity and specificity are deficient. Further evaluation of the diagnosis, staging, and assessment of resectability requires a supplemented examination. Multiphase helical computed tomography (CT) with intravenous contrast material, endoscopic ultrasound (EUS) with biopsy, aspiration cytology or both, and magnetic resonance imaging (MRI) combined with magnetic resonance cholangiopancreatography (MRCP) provide the highest sensitivity and diagnostic value (Seufferlein 2012). Contrast-enhanced CT is in general sufficient to frame an initial management plan for pancreatic lesions and predicts surgical resectability with an accuracy up to 80 to 90%
(Karmazanovsky 2005) (Figure 5). EUS is useful especially to characterize ambiguous pancreatic lesions and when pancreatic cancer is suspected, but with no detectable mass on CT, or when pathologic confirmation is required (De Angelis 2013). In the differential diagnosis of chronic pancreatitis and pancreatic cancer, positron emission tomography (PET) scanning is useful but not superior to other imaging techniques (Kauhanen 2009, Rijkers 2014). It can be applicable for differentiating between benign and malignant pancreatic cysts and for identifying distant metastases in selected cases (Murakami 2011, Kauhanen 2015). Methods such as novel pancreatic-juice biomarkers, molecular-imaging techniques, further enhancements in EUS techniques, and combinations of these, are anticipated in future to revolutionize the detection of early pancreatic cancer (Kenner 2015).

**Figure 5.** Contrast-enhanced computed tomography (CT) image of a pancreatic tumor. A CT scan typically allows initial estimation of diagnosis, resectability, and prognosis. The pancreatic tumor of 1.76 cm in diameter (I) is indicated by A.
6.5.3 Histological verification

For patients who will undergo surgery for a pancreatic tumor with suspicion of malignancy cytological or histological verification is recommended but not mandatory. Instead, histological proof of malignancy is required prior to initiation of chemotherapy or radiation therapy. If imaging techniques give equivocal results, biopsy can also be a necessary approach. In patients with jaundice, endoscopic retrograde cholangiopancreatography (ERCP) and bile-duct stenting accompanied by ductal brushing and lavage is useful when relieving bile-duct obstruction. ERCP may provide additional information on the bile- and pancreatic ducts. Otherwise, if no biliary obstruction is present, endoscopic- or transabdominal-ultrasound-guided biopsies are feasible methods to obtain a histological sample of the tumor (Ducreux 2015).

6.5.4 Tumor markers

Tumor markers are detectable biomarkers produced either by non-cancerous cells in response to cancer, or by cancer cells themselves. They are sometimes elevated also in other conditions, and most are produced in small amounts even in normal cells. Tumor markers in general must be easily determinable, for example from blood samples, body fluids, or tissue samples. In PDAC, tumor markers are analyzed at diagnosis, but are neither sufficiently sensitive nor specific to diagnose pancreatic cancer without other diagnostic methods. They predominantly serve in the follow-up of pancreatic cancer to detect recurrence or progression of the disease, but they can prove useful in differential diagnostics, with certain limitations.

In Finland, serum carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA) are the tumor markers routinely used in diagnosis, follow-up, and even in the estimation of PDAC prognosis, even though their use presents some challenges (Lundin 1994). Of these two, CA19-9 is the more specific and sensitive for PDAC (Humphris 2012). CA19-9 (also known as sialylated Lewis-A-antigen) is an antibody that binds to tumor surface-marker sialyl Lewis A (Magnani 2004). About 4 to 10% of Caucasian population are Lewis A-negative and express no CA19-9, thus, a negative CA19-9 never rules out pancreatic cancer. Its sensitivity for pancreatic cancer is 70 to 90% and its specificity 68 to 91% (Goonetilleke 2007). CA19-9 may also be highly increased in other gastrointestinal tract cancers and in other adenocarcinomas, and can be due to non-malignant conditions such as pancreatitis, cholangitis, liver cirrhosis, decompensated DM, and biliary obstruction. Further, it is relatively insensitive regarding early, small-diameter, and poorly differentiated PDACs (Duffy 2010). Although CA19-9 fails to meet the criteria for a diagnostic marker, it nevertheless proves a useful tool for PDAC assessment: prominently
increased pretreatment CA19-9 levels >100 kU/L may predict unresectable or metastatic disease, a decrease in post-treatment CA19-9 serum level is associated with prolonged survival, and its fluctuations can indicate responsiveness to chemotherapy (Ballehaninna 2012).

CEA is commonly used in diagnosis of colorectal cancer, but it can be likewise elevated in gastric and pancreatic cancers. Additionally, smoking, banana consumption, and nonspecific colitis can elevate CEA. In pancreatic cancer, its sensitivity in a recent pooled meta-analysis was 40% and its specificity 81% (Zhang 2015). Other serum markers for diagnosis of pancreatic cancer include CA125, CA242, HCG-β, tissue polypeptide antigen (TPA), macrophage inhibitory cytokine-1 (Mic-1), and insulin-like growth-factor binding protein-1 (IGFBP-1), among others, but none of these is yet in routine clinical use (Louhimo 2004, Duffy 2010, Coppin 2016). Combining several of these tumor markers enhances diagnostic specificity but reduces its sensitivity.

Although tissue tumor-markers are targets of strenuous exploration in the search for an appropriate diagnostic tool for early PDAC, unfortunately, efforts have as yet been unsuccessful. For example, KRAS and p53 mutations lack sensitivity and specificity, because either can occur in pancreatitis and in precursor lesions; some mucins and micro-RNAs have been slightly more promising. Cytokines, especially in panels combined with CA19-9, seem to be of some diagnostic value but evidence is still insufficient (Yako 2016). Thus far, the European Group on Tumor Markers status report states that the gold-standard tumor marker for patients with PDAC remains CA19-9 (Duffy 2010).
6.6 Treatment of pancreatic cancer

6.6.1 Staging and treatment strategy

Treating patients with pancreatic cancer requires a multidisciplinary team including oncologists, surgeons, radiologists, pathologists, anesthesiologists, gastroenterologists, dietitians, social workers, and experts on palliative care (Pawlik 2008). To evaluate PDAC resectability, it is staged based on CT imaging according to tumor-node-metastasis (TNM) classification. The American Joint Committee on Cancer (AJCC) has established a staging system in which stages I and II are considered resectable and stages III and IV unresectable (Table 3).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Tumor Grade</th>
<th>Nodal Status</th>
<th>Distant Metastases</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA</td>
<td>T1</td>
<td>N0</td>
<td>M0</td>
<td>Tumor limited to the pancreas, ≤2 cm in longest dimension</td>
</tr>
<tr>
<td>IB</td>
<td>T2</td>
<td>N0</td>
<td>M0</td>
<td>Tumor limited to the pancreas, &gt;2 cm in longest dimension</td>
</tr>
<tr>
<td>IIA</td>
<td>T3</td>
<td>N0</td>
<td>M0</td>
<td>Tumor extends beyond the pancreas but does not involve the celiac axis or superior mesenteric artery</td>
</tr>
<tr>
<td>IIB</td>
<td>T1-3</td>
<td>N1</td>
<td>M0</td>
<td>Regional lymph-node metastasis</td>
</tr>
<tr>
<td>III</td>
<td>T4</td>
<td>N0/N1</td>
<td>M0</td>
<td>Tumor involves the celiac axis or the superior mesenteric artery, no distant metastasis (unresectable disease)</td>
</tr>
<tr>
<td>IV</td>
<td>T1-4</td>
<td>N0/N1</td>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
</tbody>
</table>

N denotes regional lymph nodes, M distant metastases, and T primary tumor.


For patients with local stage I and II resectable pancreatic cancer, surgery is the treatment of choice, if the patient is otherwise fit for that large an operation (Carpelan-Holmstrom 2005, Shaib 2007). Even in the elderly, age alone should not be contraindicative for pancreatic resection (Riall 2009). Centralization of pancreatic surgeries into high-volume hospitals is beneficial to patient outcome with fewer complications and lower mortality rates (Nordback 2002, Gooiker 2011, de Wilde 2012, Mamidanna 2016).
The International Study Group of Pancreatic Surgery (ISGPS) defines borderline resectable pancreatic cancer in their consensus statement according to the National Comprehensive Cancer Network’s (NCCN) previous recommendations (Bockhorn 2014, National Comprehensive Cancer Network) (Table 4). Generally, in locally advanced pancreatic cancer, venous resections should not be considered contraindicative if the patient is otherwise suitable for pancreatic surgery. In contrast, arterial resection must be considered more individually; it may be justifiable in selected cases, but predominantly, patients with arterial infiltration should be initially treated with neoadjuvant therapy and afterwards be reevaluated (Seufferlein 2012, Bockhorn 2014).

<table>
<thead>
<tr>
<th>Localized and resectable</th>
<th>Borderline resectable</th>
<th>Unresectable*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No distant metastasis</td>
<td>No distant metastasis</td>
<td>Distant metastasis</td>
</tr>
<tr>
<td>No radiographic evidence of SMV or PV distortion</td>
<td>Venous involvement of the SMV or PV with distortion or narrowing of the vein or occlusion of the vein with suitable vessel proximal and distal, allowing for safe resection and replacement.</td>
<td>Greater than 180° SMA encasement, any celiac abutment, IVC</td>
</tr>
<tr>
<td>Clear fat planes around CA, HA, and SMA</td>
<td>GA encasement up to the hepatic artery with either short segment encasement or direct abutment of the HA without extension to the CA</td>
<td>Unreconstructible SMV/portal occlusion</td>
</tr>
<tr>
<td></td>
<td>Tumor abutment of the SMA not to exceed 180° of the circumference of the vessel wall.</td>
<td>Aortic invasion or encasement</td>
</tr>
</tbody>
</table>

*Criteria are given only for carcinomas of the head.

Abbreviations: CA, Celiac axis; GA, gastroduodenal artery; HA, hepatic artery; IVC, inferior vena cava; NCCN, National Comprehensive Cancer Network; PV, portal vein; SMA, superior mesenteric artery; SMV, superior mesenteric vein


Following the CT, with a pancreatic-tumor protocol and initial evaluation of resectability, several factors still remain for consideration. Approximately 70% of all patients with pancreatic cancer develop jaundice due to biliary obstruction which can be relieved by endoscopic stenting via ERCP, during
which it is also recommendable to take a cytology sample. Preoperative biliary decompression is not routinely advisable if it delays surgery in resectable pancreatic cancer, unless the patient has cholangitis or needs other preoperative evaluation or treatment delaying surgery (van der Gaag 2010, Fang 2012). In locally advanced and metastatic disease (unresectable), ERCP is recommended if otherwise possible, but this can be limited by comorbidity (Boulay 2014). Even though a pancreatic tumor is staged as resectable by the imaging techniques, guidelines still encourage staging laparoscopy to avoid unnecessary laparotomy, especially in large tumors and in cases with high CA19-9, because distant metastases are discovered during surgery in 10 to 20% (Seufferlein 2012, Allen 2013, Schnelldorfer 2014).

Patients with technically unresectable or borderline resectable pancreatic cancer may benefit from an attempt to downsize the tumor with neoadjuvant chemotherapy or chemoradiotherapy (Gillen 2010). When successful, neoadjuvant therapy converts the tumor to resectable. In contrast, no patient whose cancer progresses during neoadjuvant treatment is a candidate for surgery (Callery 2009, Seufferlein 2012). Metastatic pancreatic cancer is always considered unresectable and incurable, although selected cases receive palliative surgery. In unresectable disease, the aim of any treatment is extension of survival and palliation of symptoms.

6.6.2 Pancreatic surgery

Radical surgery in combination with oncological treatment remains the only PDAC cure. Only approximately 10 to 20% of the patients are, at diagnosis, candidates for surgical treatment (Seppänen 2016, Siegel 2016). Surgery extends the median survival time of such patients from 6 months (all stages at diagnosis) to 11 to 24 months (Hidalgo 2010, Vincent 2011). The 5-year survival of surgically treated pancreatic cancer ranges from 7 to 25% (Seppänen 2016, Cancer Research UK). Although pancreatic surgery is invasive and demanding, perioperative mortality is relatively low, especially in high-volume centers, ranging from 1 to 4% (Hartwig 2016, Mamidanna 2016, Seppänen 2016). The major aim of the operation is total removal of the tumor with radical (R0) ≥1 mm margins; this is achievable in 26 to 74% (Chandrasegaram 2015).

The majority of pancreatic tumors localize in the head of the pancreas, in which case the surgery is generally pancreaticoduodenectomy either by a pylorus-preserving or standard procedure (Whipple 1935) (Figure 6). Distal (left) pancreatic resection including splenectomy, is often feasible approach if the tumor is situated in the pancreatic body or tail, although these sometimes require total pancreatectomy (Tol 2014).
According to the ISGAPS consensus statement in 2014, the standard lymphadenectomy during pancreaticoduodenectomy comprises the lymph nodes in the anterosuperior group along the common hepatic artery, along the bile- and the cystic duct, the posterior and anterior groups of the head of the pancreas, and those along the right lateral superior mesenteric artery, as well as the suprapyloric and infrapyloric lymph nodes (Tol 2014). Furthermore, the lymphadenectomy should include ≥15 lymph nodes, and the pathologist should report the lymph-node ratio (LNR, metastatic lymph nodes divided by total number of lymph nodes examined) in the analysis. Neither extended lymphadenectomy, pylorus preservation, more extensive surgery, nor minimally invasive techniques seem to provide any survival benefit (Hartwig 2013, Ryan 2014, Dasari 2015, Ricci 2015). Unfortunately, after surgical resection, patients often develop systemic (>70%) and local recurrence (>20%) (Hidalgo 2010). Yet, due to improved oncological treatment, reoperation after PDAC recurrence and even resection of treatment-responding metastases may in a few carefully selected cases improve patient outcome (Miyazaki 2014, Chang 2016, Crippa 2016).
6.6.3 Oncological treatment

Oncoological treatment includes chemotherapy and radiotherapy administered pre- or post-operatively or in advanced disease. The aim of neoadjuvant therapy, administered prior to surgery, is to convert primarily unresectable disease to resectable by tumor downsizing. Post-operational adjuvant therapy reduces risk for recurrence and improves patient outcome. The aim of palliative therapy is to prolong life expectancy in metastatic and unresectable disease. However, high resistance to systemic therapies is characteristic of PDAC, and chemotherapy alone cannot be expected to be curative (Ryan 2014).

5.6.3.1 Neoadjuvant therapy

Neoadjuvant therapy with gemcitabine (sometimes in combination with another cytostatic) or FOLFIRINOX (a chemotherapy regimen combining folinic acid, fluorouracil, irinotecan, and oxaliplatin), followed by chemoradiation if suitable, is considered if the tumor is local but primarily borderline resectable or unresectable (Ducreux 2015). A good response to neoadjuvant treatment converts the tumor to resectable in some 30 to 60% of cases and improves the outcome of patients with borderline resectable PDAC so it becomes close to the outcome of patients with primarily resectable disease (Gillen 2010, Assifi 2011, Andriulli 2012). According to a meta-analysis in 2010, survival of patients with resectable pancreatic cancer is unaffected by neoadjuvant therapy; it should be the choice only for primarily borderline resectable disease (Gillen 2010). Currently, several randomized multi-center studies are prospectively assessing potential neoadjuvant treatment regimens (Ducreux 2015, Shaib 2016).

5.6.3.2 Adjuvant therapy

The current recommendation for all patients undergoing surgery for PDAC is 6 months (including six cycles) of postoperative adjuvant chemotherapy with either gemcitabine or 5-fluorouracil (5-FU), beginning approximately 12 weeks after surgery (Neoptolemos 2010, Liao 2013, Oettle 2013, Valle 2014). Adjuvant therapy improves both disease-free survival and overall survival with gemcitabine and 5-FU equally. Adjuvant chemoradiotherapy, frequently used in the USA but rarely in Europe, plays a more controversial role, and its benefit is still under debate (Neoptolemos 2001, Liao 2013, Neuzillet 2015).
5.6.3.3 Palliative therapy

For advanced PDAC, the only validated treatment until 2010 was gemcitabine, but PDAC is highly resistant to chemotherapy; only approximately 15% respond to gemcitabine in advanced disease (Hashimoto 2009). Furthermore, gemcitabine improves median survival time of advanced PDAC rather modestly, from 3 months (with the optimal supportive care) to 5 to 6 months. Recent data indicate that the expression of a membrane transporter, human equilibrative nucleoside transporter 1 (hENT1), is associated with responsiveness to gemcitabine and also with overall survival; it could potentially prove useful in prognostic evaluation (Nordh 2014).

Combining nab-paclitaxel with gemcitabine improves median survival time to 8.5 months (Von Hoff 2013), and the combination regimen FOLFIRINOX extends median survival time to 11.1 months (Conroy 2011). The disadvantage of combination therapies is their higher toxicity, so only those patients with good performance status are eligible. Single-gemcitabine remains the treatment of choice for patients >75 years of age or with moderate performance status. Currently, the combination therapies undergo study also in neoadjuvant and adjuvant settings (Neuzillet 2015).
6.7 Prognostic evaluation

6.7.1 Patient-related prognostic factors

The incidence of pancreatic cancer increases progressively with age. In most studies, the prognosis is unaffected by age alone, although increasing age often brings more co-morbidities, which, in turn, can affect survival (Riall 2009, Casadei 2014, Teague 2015). Pancreatic cancer is slightly more common in men than women, but few studies concern gender’s association with prognosis; the few that exist show no significant correlation (Molife 2001). Simultaneous diabetes mellitus (mainly type 2), being common in patients with pancreatic cancer, seems to be associated with worse survival, especially in surgically treated patients (Li 2012, Toriola 2014).

6.7.2 Tumor-related prognostic factors

In pancreatic cancer, the key determinants of prognosis are tumor stage and resectability (Table 3). If the tumor is unresectable, the disease is incurable, and expected survival time is only a few months, increased marginally by chemotherapy.

Once the tumor is resected, several prognostic factors can be determined from the resected preparate. One of the critical factors is radicality of the resection: an >1 mm resection margin of tissue without tumor infiltration (R0). Other well-established prognostic factors include tumor size, tumor differentiation grade, lymph-node metastasis (N0/N1), as well as neural, lymph-vessel and vascular tumor invasion (Gebhardt 2000, Garcea 2008, Benassai 2015). Further, lymph-node involvement alone is not as important in the prognosis as is the LNR; a high LNR (number of metastatic lymph nodes divided by total number of lymph nodes analyzed >0.2) is an important independent negative prognostic factor (Riediger 2009, Huebner 2012, Robinson 2012, Valsangkar 2013).
6.7.3 Prognostic serum tumor markers and tissue markers

Several biomarkers detectable either in the peripheral blood circulation or in the tumor mass correlate with PDAC prognosis. Serum tumor markers of PDAC, including CA19-9, CEA, CA242, and CA72-2, have prognostic potential particularly in advanced disease based on their dynamics in response to chemotherapy (Louhimo 2004, Boeck 2006, Goonetilleke 2007, Ballehaninna 2012, Humphris 2012, Liu 2015, Reitz 2015). CA19-9 and CEA predict prognosis also in resectable PDAC, and an increase in CA19-9 or CEA levels after treatment is indicative of disease recurrence and progression (Lundin 1994, Distler 2013, Piagnerelli 2016). Moreover, many tissue markers show prognostic potential, including PROX1, beta-catenin, podocalyxin (Saukkonen 2015, 2016), the cytoskeletal protein ezrin (Piao 2015), several members of the MMP family (Juuti 2006, Mroczko 2009, Xu 2015), and some growth factors (Yamanaka 1993, Uegaki 1997).

6.7.4 Prognostic potential of inflammatory biomarkers

Tumor-associated inflammation can lead to elevated concentrations of inflammatory biomarkers in the peripheral blood circulation. Levels of C-reactive protein (CRP) above normal are associated with worse prognosis in cancers of the breast, colon, and urological system (Han 2011, Kersten 2013, Saito 2013, Thurner 2015). A correlation between CRP and PDAC prognosis is best established in patients receiving palliative chemotherapy (Haas 2013, Mitsunaga 2016). In patients operated on with curative intent, study cohorts have been very small. A meta-analysis in 2015 noted that elevated CRP level may be independently indicative of worse prognosis in PDAC, but further studies are necessary (Stevens 2015).

The most abundant protein in the human blood circulation is albumin (Farrugia 2010); its reduced levels (hypoalbuminemia) indicate poor nutritional status, but are also related to SIR (Gabay 1999). Hypoalbuminemia is associated with poor clinical outcomes in acute illnesses (Vincent 2003) and in some cancer forms (McMillan 2001, Ataseven 2015, Chiang 2015, Kim 2015). In resectable PDAC, the effect of hypoalbuminemia on prognosis is controversial (Ruiz-Tovar 2010, La Torre 2012, Stotz 2013). Combinations of different inflammatory markers have been created to estimate cancer prognosis. Recent international guidelines recommend measurement of either the modified Glasgow prognostic score (mGPS) or the neutrophil-to-lymphocyte ratio (NLR) in all patients considered for resection of PDAC (Bockhorn 2014).
5.7.4.1 The Glasgow prognostic score

A combination of elevated CRP and low albumin levels, the Glasgow prognostic score (GPS), was originally designed in a cohort of patients with non-small-cell lung cancer for estimating their prognosis (Forrest 2003). Later, McMillan et al. (2007) modified the GPS by emphasizing the elevated CRP, because, in some studies, hypoalbuminemia was not an independent predictor of survival. In one large retrospective cohort study, the modified Glasgow prognostic score (mGPS) predicted cancer survival independent of tumor site (Proctor 2011). Table 5 shows how the GPS and the mGPS are determined (Proctor 2011, Forrest 2003).

Table 5. The Glasgow prognostic score (GPS) and modified Glasgow prognostic score (mGPS) calculated with C-reactive protein (CRP) and albumin.

<table>
<thead>
<tr>
<th>Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GPS</strong></td>
<td>Normal CRP &lt;10 mg/L AND Normal albumin &gt;35 g/L</td>
<td>Elevated CRP &gt;10 mg/L OR Hypoalbuminemia &lt;35 g/L</td>
<td>Elevated CRP &gt;10 mg/L AND Hypoalbuminemia &lt;35 g/L</td>
</tr>
<tr>
<td><strong>mGPS</strong></td>
<td>Normal CRP &lt;10 mg/L AND Irrespective of albumin</td>
<td>Elevated CRP &gt;10 mg/L AND Normal albumin &gt;35 g/L</td>
<td>Elevated CRP &gt;10 mg/L AND Hypoalbuminemia &lt;35 g/L</td>
</tr>
</tbody>
</table>

5.7.4.2 Prognostic ratios based on inflammatory cells

Some inflammatory-cell-based ratios also predict cancer-patient survival. Elevated NLR has lately been recognized in several cancer forms as a simple indicator of worse prognosis (Li 2014, Paramanathan 2014, Xiao 2014, Zhang 2014, Yang 2015). NLR is, according to some recent studies, feasible also in resectable pancreatic cancer to predict postoperative survival (Stotz 2013, Stevens 2015). Furthermore, the platelet to lymphocyte ratio (PLR) shows some prognostic potential in malignancies including PDAC (Koh 2015, Shirai 2015, Spolverato 2015), but in significance PLR loses out to NLR (Bhatti 2010, Azab 2014, Zhang 2014).
6.8 Macrophages and pancreatic cancer

6.8.1 Polarization of macrophages: types M1 and M2

Macrophages are a type of white blood cells found in practically all human tissues. They eliminate cellular debris, microbes, and foreign substances by phagocytosis, being among the first cells to infiltrate a damaged or infected tissue. They prove critical in both innate and adaptive immunity by activating other immune cells. For decades, scientists focused on their role in immune defense, but macrophages are additionally pivotal in immune regulation and homeostasis by, for example, participating in tissue remodeling and wound healing via mechanisms independent of immune-cell signaling (Erwig 2007).

Macrophages originate from common hematopoietic stem cells in the bone marrow, where they differentiate first through the myeloid and then through the monocyte lineage. Monocytes are mobilized from the bone marrow into the blood stream; subsequently they extravasate and infiltrate target tissues both in the steady state and also in response to inflammation. In tissues, monocytes mature into tissue-specific macrophages (Gordon 2005).

Macrophages are remarkably diverse and plastic cells that respond dynamically to environmental signaling by efficiently changing their functional phenotype, which leads to alterations in their physiology (Mosser 2008). In the 1990s, scientists discovered that besides their classical activation in response to interferon (IFN) γ and toll-like receptor (TLR) agonists, there is also an alternative route to macrophage activation, detected by up-regulated expression of the mannose receptor (CD206), enhanced by Th2 cell-produced IL-4 and IL-13 (Stein 1992, Doyle 1994). The distinct types of macrophages were later named analogously to T-cell nomenclature (Th1 and Th2), as M1 and M2 macrophages (Mills 2000).

The classically activated type of M1 macrophages are pro-inflammatory; they participate actively in the host defense by being effectors of Th1 responses; they produce high amounts of inflammatory cytokines such as IL-1β, IL-6, IL-12, IL-23, and TNFα; and they mediate resistance against tumors and microbes (Mantovani 2005) (Figure 7). By definition, all other types of macrophages are subtypes of M2 macrophages, playing varying roles in tissue repair, immune regulation, and even in cancer promotion. They mediate their immune-response suppressive role by producing anti-inflammatory cytokines such as IL-10 (Mosser 2008, Sica 2012). Type M2 macrophages can be further divided into several subgroups called M2a, M2b, M2c, and M2d (Edwards 2006, Mosser 2008, Sica 2012). Besides the division of M1 and M2 macrophages by the inflammatory and anti-inflammatory cytokines they
produce, there occur several patterns of cell-surface markers for macrophage characterization. Cell-surface markers that are characteristic of M1 macrophages include CD80 and CD86, whereas characteristic for M2 macrophages are CD206 (mannose receptor), CD209, and CD163 (scavenger receptor). CD14 is expressed in most macrophages but is especially abundant in TAMs. This division is, however, a simplification, and in reality, macrophage polarization is much more complex; M1 and M2 macrophages are only the two extremes of the spectrum of macrophage polarization (Mosser 2008, Sica 2013).

Due to their plasticity, macrophages can efficiently change their M1/M2 polarization in response to environmental stimuli. Moreover, macrophages autoregulate themselves by forming positive feedback loops in which the inflammatory cytokines produced by M1 and the anti-inflammatory ones produced by M2 cells further stimulate their polarization to shift towards their respective phenotypes (Mantovani 2007). Under in vivo conditions often simultaneously multiple opposing reprogramming signals operate in macrophage microenvironment. The fact that researchers have been able to induce, reverse, and re-polarize macrophage activation promises important therapeutic implications (Sica 2012, Zhou 2014).
Figure 7. Simplified view of macrophage polarization to types M1 and M2. In the presence of microbial and Th1 cell products such as IFNγ and LPS, monocytes differentiate preferentially into M1 macrophages that produce inflammatory mediators and enhance the inflammatory response. IL-4 and other anti-inflammatory cytokines skew macrophage polarization toward type M2 macrophages that in turn produce immunoregulatory cytokines. In reality, macrophage polarization is a continuum with both inflammatory and immunoregulatory properties. Illustration by the author.
6.8.2 Tumor-associated macrophages

Macrophages are in cancerous tumors a major stromal component (Feig 2012). Chemokines, cytokines, and products of the complement cascade recruit circulating monocytes to the tumor site (Franklin 2014, Noy 2014). In the proximity of tumor cells macrophages, differentiate into type M2d macrophages, also referred to as tumor-associated macrophages (TAMs) (Sica 2012). In tumors, TAM abundance is correlated with immunosuppression, tumor growth, neovascularization, invasiveness, metastasis, and resistance to therapy (Qian 2010). TAMs show various tumor-promoting functions; for example, they support tumor vascularization by producing pro-angiogenic factors such as vascular endothelial growth factors (VEGFs). They also suppress anticancer-immune responses, for example the cytotoxicity of CD8+ T cells. Further, TAMs modulate and degrade the extracellular matrix (ECM) through such products as secreted protein acidic and rich in cysteine (SPARC) and MMPs aiding cancer cells to invade the blood vessels and thus metastasize (Puolakkainen 2004, Arnold 2010, Kitamura 2015, Shen 2016).

An important signaling pathway in pancreatic cancer cells and macrophages seems to be the STATs (signal transducers and activators of transcription), a family of transcription factors activated by tyrosine phosphorylation of Janus-activated kinases (JAKs). Several cytokines and growth factors can bind to JAKs, and these transfer extracellular signals via STATs to the cell nucleus, where STATs bind to promoter sequences and activate gene transcription (Hu 2007). The JAK/STAT pathway is responsible for the activation of thousands of protein-encoding genes, including those important for cell proliferation and macrophage differentiation; it is therefore not surprising that several types of tumors display aberrant STAT activation (Toyonaga 2003, Palagani 2014, O'Shea 2015). In macrophages, the STAT 1 pathway is predominantly activated in M1, but STAT 3 and 6 are activated in type M2 (Porta 2015).

During cancer progression, the TAM phenotype changes and varies even in the tumor tissue itself. In tissues with chronic inflammation and initial cancer development, primarily M1 macrophages occur, whereas M2 macrophages are more abundant at later stages when the tumor begins neovascularization and invasion (Mielgo 2013). Complex signaling from tumor- and host cells further shape TAMs’ functional phenotypes. Cancer cells generate cytokines such as TGFβ and Colony-Stimulating Factor-1 (CSF-1, also known as macrophage-colony-stimulating factor, M-CSF) and metabolic products (lactic acid among others) that polarize TAMs. Further, participating in this functional shaping of TAMs are a hypoxic microenvironment, the Th2 cell products IL-4 and IL-13, regulatory T-cell-produced IL-10, and immune complexes from B cells (Sica 2012, Noy 2014).
TAMs also orchestrate many mechanisms of resistance to chemotherapy, mainly by misdirected tissue repair-responses, for example by recruitment of more immunosuppressive TAMs, by skewing macrophage polarization towards pro-tumor functions, and by protecting cancerous cells against chemotherapy (Mantovani 2015). All this makes macrophages a tempting area for development of new anticancer therapies and encourages scientists to explore macrophages further (Baay 2011, Noy 2014, Ostuni 2015).

6.8.3 Tumor-associated macrophages in pancreatic cancer

Pancreatic cancer is characteristically rich in stroma and inflammatory cells, including TAMs (Feig 2012). In PDAC, TAMs play a critical role in tumor progression; abundance of TAMs is associated with worse prognosis and less responsiveness to chemotherapy (Kurahara 2011, Yoshikawa 2012, Hou 2014, Di Caro 2015). Analogous to other types of solid tumors, the tumor-promoting effects of TAMs in PDAC include supporting tumor vascularization, degrading the ECM, and suppressing immune reactions and tumor-resistant actions (Mielgo 2013). Further, M2 TAMs are, at least in part, responsible for resistance to gemcitabine, whereas recruitment of type M1 macrophages enhances gemcitabine efficacy (Beatty 2011, Weizman 2014). Some new data even suggest that TAMs may mediate the pancreatic-cancer-suppressive effects of metformin (Soranna 2012, Incio 2015).

Although macrophages are amply present also in chronic pancreatitis, they are typically of type M1; in pancreatic cancer, macrophages are polarized more towards the immune-response suppressive M2 phenotype (Mielgo 2013, Helm 2014, Chen 2015). High levels of pro-inflammatory cytokines (for example TNFα, IL-1β) in pancreatitis recruit monocytes and macrophages to the pancreas, subsequently generating an inflammatory microenvironment (Sah 2012). In pancreatitis, neutralization of the acute inflammation leads to less disease severity, and the transition of M1 to M2 is actually therefore favorable (Perides 2011, Sah 2012). In contrast, in PDAC the M2 type in particular seems to correlate with worse survival; therefore efforts focus on converting macrophages in PDAC tumors to the anti-tumor M1 type (Kurahara 2011, Mielgo 2013).
6.8.4 *In vitro* polarization of macrophages

Macrophages for *in vitro* studies originate predominantly from either murine bone marrow or human monocytes isolated from peripheral blood. Discovery of an alternative macrophage activation route with IL-4 (in contrast to classical activation with IFNγ) has generated a wave of studies on macrophage polarization. Earlier, researchers have used granulocyte-macrophage colony-stimulating factor (GM-CSF) to generate inflammatory M1 macrophages and macrophage colony-stimulating factor (M-CSF, also called CFS-1) to generate anti-inflammatory M2 macrophages (Fleetwood 2007). The cell cultures are often supplemented with various cytokines to further stimulate the macrophage activation. For example, IFNγ and LPS are typical choices for the classical, inflammatory activation, whereas IL-4 and IL-13 serve as activators for the alternative route (Stein 1992, Doyle 1994, Mills 2000).

The *in vitro* polarization and study of macrophages has raised some criticism. The nomenclature and methods are diverse, and therefore not always directly comparable. In a consensus study, Murray *et al.* (2014) emphasize the extreme plasticity of macrophages and suggest naming the *in vitro*-differentiated macrophages according to the stimulants used, as compared to M1 or M2 macrophages. In the *in vitro* setting, monocytes are first isolated and then differentiated separate from their natural surroundings without interaction with other immune cells and stromal elements. Some scientists, like Nahrendorf *et al.* (2016), suggest abandoning the M1/M2 distinction altogether to avoid an oversimplified view, and recommend thinking of macrophages as a network of immune cells and referring to them according to their function in their natural habitat.
Aims of the present study

7 AIMS OF THE PRESENT STUDY

The main objective of this study was to create further insights into the nature of pancreatic cancer and its association with inflammation, with emphasis on the role of tumor-associated macrophages (TAMs). We explored the association between TAMs and the migration of pancreatic cancer cells and the changes that occur in macrophages under the influence of pancreatic cancer cells. More specifically the aims were to:

1. study the role of MMP9, ADAM8, and hypoxia in pancreatic cancer cell invasion in co-cultures with macrophages

2. polarize macrophages into types M1 and M2 and assess their impact on pancreatic cancer cell migration

3. investigate the possibilities of modifying pancreatic cancer cell migration through macrophage polarization

4. assess the role of STAT activation in pancreatic cancer cell invasion in co-cultures with macrophages

5. study the association of systemic inflammatory response with the adverse effect on the prognosis of patients with surgically treated pancreatic cancer
8 MATERIALS, PATIENTS, AND METHODS

8.1 Ethical statement

All studies followed the Declaration of Helsinki and had the approval of the Helsinki University Hospital Ethics Committee. Furthermore, Study IV had the approval of the National Supervisory Authority for Welfare and Health (Valvira). All healthy blood donor volunteers were informed about the study procedures. Patients who prospectively gave blood samples for Study IV signed a written informed consent. The guidelines of Helsinki University Hospital and National Data Protection Commission assured the confidentiality of healthy subjects’ names and all patient information.

8.2 Cell lines and cultures

The source of macrophages was the isolation and differentiation of monocytes from healthy human donors’ peripheral blood samples. The blood sample from each donor was processed and assayed separately. First, mononuclear cells were separated by density gradient centrifugation with Ficoll-Paque Plus (Amersham, Uppsala, Sweden). Monocytes were further isolated by negative paramagnetic bead separation with the Human Monocyte Isolation Kit II (Miltenyi Biotec, Auburn, TX, USA) according to manufacturer’s instructions for Studies II and III. Differentiation into macrophages was initiated directly after monocyte isolation and continued for 5 days before addition of pancreatic cancer cells or additional stimuli or both (Figure 8).
Materials, patients, and methods

Figure 8. Isolation of monocytes, their differentiation into macrophages, and preparation for assays. (Studies I-III)
The human pancreatic adenocarcinoma cell lines MiaPaCa-2, PANC-1 (primary tumor cell lines), HPAF-II, and ASPC-1 (metastatic tumor cell lines) came from the American Type Culture Collection (ATCC). Those pancreatic adenocarcinoma cells not used in the assays were cultured and handled according to the recommendation of the distributor. Cancer cells were added to macrophage cultures after 5 days of monocyte isolation.

For the assays, both macrophages and pancreatic adenocarcinoma cells were cultured in Macrophage serum-free media (Macrophages-SFM, Gibco Life Technologies, Paisley, UK) supplemented with penicillin (Sigma, St. Louis, MO, USA) 100 mg/ml, and either GM-CSF (ImmunoTools, Oldenburg, Germany) 10 ng/ml or M-CSF (ImmunoTools) 50 ng/ml to differentiate the isolated monocytes into mature M1 and M2 macrophages, respectively. All cells were incubated in standard +37 ºC and 5% CO2. The additional stimuli were added after 5 days of monocyte differentiation (Figure 8). In Table 6 are presented all the supplements and their concentrations added to the cell cultures.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Name</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>stimulating factor</td>
<td>10 ng/ml</td>
<td>ImmunoTools, Oldenburg, Germany</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
<td>50 ng/ml</td>
<td>ImmunoTools</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
<td>50 ng/ml</td>
<td>R&amp;D Systems, Shanghai, China</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin 4</td>
<td>20 ng/ml</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
<td>10 ng/ml</td>
<td>Sigma, St. Louis, MO, USA</td>
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<td>IL-12</td>
<td>Interleukin 12</td>
<td>5 ng/ml</td>
<td>R&amp;D Systems</td>
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<td>IL-10</td>
<td>Interleukin 10</td>
<td>25 ng/ml</td>
<td>R&amp;D Systems</td>
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<tr>
<td>P6</td>
<td>Pyridone 6</td>
<td>500 nM</td>
<td>Calbiochem, San Diego, CA, USA</td>
</tr>
</tbody>
</table>

Table 6. Supplementary stimulants and inhibitors.
8.3 Cancer cell migration studies

After 5 days of macrophage differentiation, the pancreatic cancer cells were stained with fluorescent dye (CellTracker Green CMFDA, Invitrogen, Eugene, OR, USA) and added to the macrophage cultures. As controls the PDAC cells were alternatively cultured alone under otherwise identical conditions. The supplementary stimulants (interleukins and P6) were also added at this stage. The cells were cultured on Matrigel (BD Biosciences, San Jose, CA, USA) simulating the ECM of the tumor microenvironment in which cells can migrate and form three-dimensional structures. The cancer cells settled for 24 hours, and then their migration rate was recorded by imaging their movement in a fluorescence microscope equipped with a cooled CCD camera (Sensicam, PCO, Kelheim, Germany) with 30-min intervals for 24 hours in a humidified temperature (+37 ºC) and CO2 (5%) -controlled chamber (OKOlab, Ottaviano, Italy). In Study I, we led a hypoxic gas mixture (5% CO2, 2% O2, N2 94%, Woikoski, Järvenpää, Finland) into the chamber to allow us to observe the migration rate in hypoxic conditions.

8.4 Flow cytometry

We used flow cytometry to detect the expression of several surface proteins and intracellular STAT phosphorylation of macrophages and cancer cells. To cause as little damage to the cells as possible, the cells were cultured on Nunc UpCell dishes (Thermo Fisher Scientific, Roskilde, Denmark) which release the cells adhered to their surfaces when the temperature of the dish is lowered from +37 ºC to room temperature. For the labeling antibodies for flow cytometry studies, see Table 7.

To study the surface expression of macrophages (Studies II and III), we first separated them from the cancer cells by magnetic bead separation with anti-CD 11b MicroBeads (Miltenyi Biotec). Subsequently, we labeled the separated macrophages with selected antibodies (CD14, CD16, CD80, CD86, CD163, CD206, CD209) (Table 7) for 20 minutes at room temperature.

In Study III, we studied STAT phosphorylation as well as NFkB and AKT phosphorylation in both macrophages and MiaPaCa-2. After their detachment from culture dishes, BD Phosflow Lyse/Fix Buffer (BD Biosystems, San Diego, CA, USA) stabilized the cells at +37 ºC for 10 minutes. Consequently, BD Perm Buffer (BD Biosystems) permeabilized the cells at -20 ºC for 30 minutes. Finally, we added the antibodies for CD45 (a lymphocyte marker to identify macrophages during analysis), for STAT 1, 3, 5, and 6 and for NFkB and AKT
(Table 7) and incubated them at room temperature for 20 minutes. We verified the specific signal pathway activation in macrophages by stimulation with TNFα (10 ng/ml, 10 min, for stimulation of NFkB), IL-6 (100 ng/ml, 5 min, for stimulation of STAT 1 and 3), IL-4 (10 ng/ml, 10 min, for stimulation of STAT 5 and 6), and LPS (100 ng/ml, 10 min, for stimulation of AKT) prior to addition of the Lyse/Fix Buffer.

After our labeling of the cells, they were assessed on FACS Calibur (CellQuest Pro software; BD Bioscience) flow cytometer, and WinMDI (v2.8) software analyzed the data. When assessing the phosphorylation data, we separated the results of macrophages and MiaPaCa-2 cells by separating them into CD45-positive and -negative cells.

### Table 7. The labeling antibodies for flow cytometry.

<table>
<thead>
<tr>
<th>Characterization of macrophages</th>
<th>Detecting</th>
<th>Name</th>
<th>Amount</th>
<th>time</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC Control</td>
<td>APC Mouse IgG1 κ Isotype Control</td>
<td>20 μL</td>
<td>20 min</td>
<td>BD Pharmingen</td>
<td></td>
</tr>
<tr>
<td>FITC Control</td>
<td>FITC Mouse IgG1 κ Isotype Control</td>
<td>20 μL</td>
<td>20 min</td>
<td>BD Pharmingen</td>
<td></td>
</tr>
<tr>
<td>PE Control</td>
<td>PE Mouse IgG1 κ Isotype Control</td>
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<td>20 min</td>
<td>BD Pharmingen</td>
<td></td>
</tr>
<tr>
<td>CD14</td>
<td>FITC Mouse Anti-Human CD14</td>
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<td>20 min</td>
<td>BD Pharmingen</td>
<td></td>
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<tr>
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<td>FITC Mouse Anti-Human CD14</td>
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<td>CD80</td>
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<tr>
<td>CD209</td>
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<table>
<thead>
<tr>
<th>STAT activation (Study III)</th>
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<th>time</th>
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<tr>
<td>STAT1</td>
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<tr>
<td>STAT3</td>
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<tr>
<td>STAT5</td>
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<td>10 μL</td>
<td>30 min</td>
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<td>STAT6</td>
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<td>NFkB</td>
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<td></td>
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</tbody>
</table>
8.5 Detection of protein and cytokine expression and secretion

In Study I we detected the gene expression of ADAM8, ADAM9, ADAM10, ADAM17, MMP2, MMP9, TIMP1, and TIMP3 by real-time PCR according to manufacturer’s instructions (TaqMan Gene Expression Assay, Thermo Fisher Scientific). First, we sorted the cells into CD14-positive and CD11b-positive macrophages and cancer cells using Miltenyi MACS© Column Technology (anti-CD14 and anti-CD11b MicroBeads, Miltenyi Biotec).

Profiling of secreted cytokines in cell cultures we assessed with Human Cytokine Array Panel A (ARY005, Proteome Profiler Arrays, R&D Systems, Minneapolis, MN, USA) in Study II and with infrared Human Q-Plex™ Custom Assay (#107749GR, Quansys Biosciences, Logan, UT, USA) in Study III. In order to detect the small changes in the cytokine profiles, in Study II the media were concentrated from 1.5ml by 3K filters (Amicon Ultra Centrifugal Units, Millipore Corporation, Billerica, MA, USA). Odyssey infrared imager (LI-COR Biosciences, Lincoln, NE, USA) imaged both assays. Odyssey Software (LI-COR Biosciences) and Q-View™ Software (Quansys Biosciences) analyzed the dot blots in Studies II and III, respectively.

8.6 siRNA

The ADAM8 and MMP9 expression in pancreatic cancer cells was down-regulated by the siRNA Transfection Kit (sc-45064, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in Study I. According to the manufacturer’s instructions, we mixed MMP9 siRNA, ADAM8 siRNA, or non-targeting negative control duplex (sc-41406, sc-29400, sc-37007, Santa Cruz Biotechnology), and transfection reagent (sc-29528, Santa Cruz Biotechnology) with transfection medium (sc-36868, Santa Cruz Biotechnology). We added 10% fetal bovine serum (FBS) after 7 h, and incubated the cells for an additional 48 h. Subsequently, we replaced the transfection medium with normal PDAC cell culture medium and 10% FBS, and used the cancer cells in migration measurements.
8.7 Western blotting

For protein analysis in Study I, macrophages and the pancreatic cancer cells were first sorted as described above. Consequently, we homogenized the sorted cells with lysis buffer (Roche, Mannheim, Germany), and determined the protein concentrations of the samples by colorimetric Bradford assay. We diluted the protein extracts in Laemmli sample buffer, incubated them for 5 min at +95 °C, and then resolved them in 10% polyacrylamide gels in Tris–glycine–SDS buffer. For analysis, we transferred the gels to nitrocellulose membranes and blocked them in Odyssey blocking buffer (927–40,000, LI-COR Biosciences). As antibodies, we used rabbit anti-ADAM8 (1:500 v/v, AB19017, Millipore, Temecula, CA, USA), rabbit anti-MMP9 (1:400 v/v, 10375-2-AP, Proteintech Group, Chicago, IL, USA), mouse anti-GAPDH (1:1,000 v/v, sc-32233, Santa Cruz Biotechnology), goat anti-rabbit IgG IRDye 800 (1:15,000 v/v, 926-31220, LI-COR Biosciences), and goat anti-mouse IgG Alexa 680 dye (1:15,000, A21058, Molecular Probes, Eugene, OR, USA). Odyssey infrared imager (LI-COR Biosciences) scanned the protein bands on the membranes.

8.8 Patients for Study IV

Study IV included retrospectively all the consecutive 265 patients operated on with curative intent for pancreatic ductal adenocarcinoma at Helsinki University Hospital from 1 January 2000 to 30 March 2013. Table 8 displays patient characteristics. We excluded patients who died of surgery-related complications, had metastatic spread, emergency surgery, or at the time of the surgery had infection, an auto-immune disease, or were on immune-suppressive medication. Patients were treated according to national guidelines. Data on clinico-pathological features and laboratory findings came from patient records, except for the CRP, which was determined from preoperatively (prospectively for study purposes) -collected plasma samples of 230 patients. The samples had been stored in freezers at -80 °C. Minimum follow-up time was 2 years, with the end of follow-up on 30 March 2015. Study IV applied the Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK) (Altman 2012).
Table 8. Patient characteristics and survival time (months). Median survival time was assessed with Kaplan-Meier analysis and significance with log-rank test. (Study IV)

<table>
<thead>
<tr>
<th></th>
<th>Median survival</th>
<th></th>
<th>p (survival time)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total n=265</td>
<td>n (%)</td>
<td>time (95% CI)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>150 (56.6)</td>
<td>25.4 (18.9-31.9)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>115 (43.4)</td>
<td>26.9 (23.8-30.0)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>70 (26.4)</td>
<td>27.4 (19.8-35.0)</td>
<td></td>
</tr>
<tr>
<td>60-64</td>
<td>57 (21.5)</td>
<td>25.7 (17.4-34.1)</td>
<td></td>
</tr>
<tr>
<td>65-70</td>
<td>59 (22.3)</td>
<td>26.3 (16.8-35.9)</td>
<td></td>
</tr>
<tr>
<td>&gt;70</td>
<td>79 (29.8)</td>
<td>26.1 (19.2-32.9)</td>
<td></td>
</tr>
<tr>
<td>Margin involvement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R0</td>
<td>207 (78.1)</td>
<td>30.0 (25.1-35.0)</td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>42 (15.8)</td>
<td>18.3 (7.6-28.9)</td>
<td></td>
</tr>
<tr>
<td>T status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>21 (7.9)</td>
<td>27.2 (15.9-38.5)</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>64 (24.2)</td>
<td>36.0 (22.3-49.7)</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>166 (62.6)</td>
<td>22.0 (17.7-26.3)</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>8 (3.0)</td>
<td>14.7 (7.4-22.0)</td>
<td></td>
</tr>
<tr>
<td>LNR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>94 (35.5)</td>
<td>33.6 (30.4-36.8)</td>
<td></td>
</tr>
<tr>
<td>≤0.2</td>
<td>123 (46.4)</td>
<td>25.7 (17.9-33.6)</td>
<td></td>
</tr>
<tr>
<td>&gt;0.2</td>
<td>42 (15.8)</td>
<td>13.6 (7.7-19.4)</td>
<td></td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head</td>
<td>230 (86.8)</td>
<td>26.0 (21.8-30.1)</td>
<td></td>
</tr>
<tr>
<td>Body</td>
<td>19 (7.2)</td>
<td>36.0 (10.3-61.7)</td>
<td></td>
</tr>
<tr>
<td>Tail</td>
<td>13 (4.9)</td>
<td>34.1 (9.4-58.8)</td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>≤30 mm</td>
<td>135 (51.0)</td>
<td>30.1 (24.8-35.4)</td>
<td></td>
</tr>
<tr>
<td>&gt;30 mm</td>
<td>120 (45.0)</td>
<td>20.5 (15.6-25.4)</td>
<td></td>
</tr>
<tr>
<td>Preoperative chemo-/radiotherapy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>218 (82.3)</td>
<td>25.7 (20.8-30.6)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>46 (17.4)</td>
<td>30.0 (23.7-36.4)</td>
<td></td>
</tr>
<tr>
<td>Postoperative chemo-/radiotherapy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>86 (32.5)</td>
<td>21.9 (14.5-29.3)</td>
<td></td>
</tr>
<tr>
<td>Adjuvant</td>
<td>139 (52.5)</td>
<td>30.1 (24.1-36.0)</td>
<td></td>
</tr>
<tr>
<td>Palliative</td>
<td>34 (12.8)</td>
<td>23.9 (15.4-32.5)</td>
<td></td>
</tr>
<tr>
<td>Cause of death</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDAC</td>
<td>202 (76.2)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>10 (3.8)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Alive</td>
<td>53 (20.0)</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

CI, confidence interval; LNR, lymph node ratio; PDAC, pancreatic ductal adenocarcinoma
8.9 Statistics

Statistical analyses were assessed with the IBM Statistical Package for Social Sciences (SPSS) Statistics 22. We tested normality by the Kolmogorov-Smirnov test. To detect differences between continuous variables we used the nonparametric Mann-Whitney U-test, the Kruskall-Wallis test, or the Wilcoxon’s signed-rank test (paired measurements). Two-tailed Spearman’s correlations were applied to calculate associations between continuous variables. In the migration assay, confidence intervals (CI) for fold change were calculated according to the rules of error propagation as asymptotic confidence intervals. We estimated survival by Kaplan-Meier analysis and the Log-rank test. Survival was determined from time of pancreatic surgery to cancer-specific death; the patient was censored at the last follow-up date if no events had occurred. To assess the significant differences in medians of laboratory values by survival time (ordinal scale), we used the Jonckheere-Terpstra test. Multivariate modeling was conducted by means of the Cox proportional hazards model. A p value <0.05 was considered statistically significant.
9 RESULTS

9.1 Characterization of macrophages (Studies I-III)

Isolated monocytes were differentiated into macrophages with either GM-CSF or M-CSF. The polarization of GM-CSF-differentiated macrophages skewed more towards type M1, had over 50% of the cells positive to CD86 and CD206. In contrast, M-CSF-differentiated macrophages, skewed more towards M2, were over 50% positive to CD14 (p=0.007 as compared to GM-CSF-differentiated cells), CD80 (p=0.017), CD86, CD163 (p=0.001), CD206 (p=0.001), and CD209 (p=0.004). Co-culture with pancreatic cancer cells caused little change in both GM-CSF- and M-CSF-differentiated macrophages’ surface expression; only the proportion of CD16-positive cells increased in GM-CSF differentiated macrophages: from 11.2 (±2.6) to 40.6% (±5.7 SEM, p=0.002) (Table 9).

Macrophage polarization was altered with the additional stimulation. Table 9 shows the proportion of macrophages positive to the different surface markers with the different stimulations, and the comparisons to respective GM-CSF-/M-CSF-differentiated macrophages with no additional stimuli (see also Figures 9A and 9B). In summary, we were able to obtain macrophages with both inflammatory and anti-inflammatory phenotypes by different stimulations. In M-CSF-differentiated macrophages, the combination of inflammatory LPS and anti-inflammatory IL-4 caused mixed effects on surface protein expression, IL-12 skewed macrophage polarization towards the inflammatory M1 type, and IL-10 towards the anti-inflammatory M2 type (data for GM-CSF-differentiated macrophages supplemented with IL-12 are unpublished results of Salmiheimo et al.). Although having other significant effects in the cell cultures, IL-6 did not change the surface protein expression of GM-CSF differentiated macrophages.

Scavenger receptor CD163 (a widely used M2 marker) responded to the stimuli consistently parallel with the expected polarization of M1/M2, being higher in M2-polarized macrophages. It was higher in M-CSF- (M2) than in GM-CSF (M1) -differentiated macrophages (p=0.001). It was high also in macrophages stimulated with IL-10 (anti-inflammatory cytokine), and low with the inflammatory IL-12-, IL-4+LPS-, and IL-6-stimulated macrophages.
Table 9. Characterization of the differently stimulated macrophages. The values represent percentages of positive cells. *p* values in this table are as compared to the respective macrophages (with or without MiaPaCa-2) without the added stimulant (for example M-CSF + IL-10 and MiaPaCa-2 is compared to M-CSF and MiaPaCa-2 macrophages).

<table>
<thead>
<tr>
<th>Macrophages with:</th>
<th>M1 markers</th>
<th></th>
<th></th>
<th>M2 markers</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD16 (SEM)</td>
<td>p (CD16)</td>
<td>CD80 (SEM)</td>
<td>p (CD80)</td>
<td>CD86 (SEM)</td>
<td>p (CD86)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>11.2 (2.6)</td>
<td>0.073</td>
<td>32.1 (10.2)</td>
<td>0.073</td>
<td>37.8 (10.5)</td>
<td>0.620</td>
</tr>
<tr>
<td>GM-CSF and MiaPaCa-2</td>
<td>40.6 (5.7)</td>
<td>0.535</td>
<td>60.8 (9.2)</td>
<td>0.902</td>
<td>49.1 (12.4)</td>
<td>0.805</td>
</tr>
<tr>
<td>GM-CSF + IL-6</td>
<td>24.5 (5.7)</td>
<td>0.073</td>
<td>53.3 (8.0)</td>
<td>0.073</td>
<td>91.5 (3.1)</td>
<td>0.620</td>
</tr>
<tr>
<td>GM-CSF + IL-6 and MiaPaCa-2</td>
<td>32.4 (6.6)</td>
<td>0.535</td>
<td>65.0 (10.3)</td>
<td>0.902</td>
<td>90.6 (3.5)</td>
<td>0.456</td>
</tr>
<tr>
<td>GM-CSF + IL-10</td>
<td>22.2 (5.7)</td>
<td>0.149</td>
<td>48.4 (10.3)</td>
<td>0.343</td>
<td>51.3 (14.5)</td>
<td>0.030</td>
</tr>
<tr>
<td>GM-CSF + IL-10 and MiaPaCa-2</td>
<td>52.6 (5.5)</td>
<td>0.106</td>
<td>86.4 (3.3)</td>
<td>0.149</td>
<td>85.1 (4.9)</td>
<td>0.073</td>
</tr>
<tr>
<td>GM-CSF + IL-12</td>
<td>8.6 (4.7)</td>
<td>0.648</td>
<td>49.9 (4.5)</td>
<td>0.109</td>
<td>95.9 (1.9)</td>
<td>0.788</td>
</tr>
<tr>
<td>GM-CSF + IL-12 and MiaPaCa-2</td>
<td>37.2 (10.1)</td>
<td>0.432</td>
<td>80.1 (3.3)</td>
<td>0.202</td>
<td>95.0 (1.5)</td>
<td>1.000</td>
</tr>
<tr>
<td>M-CSF</td>
<td>21.2 (6.2)</td>
<td>0.073</td>
<td>62.0 (4.7)</td>
<td>0.073</td>
<td>91.8 (2.7)</td>
<td>0.620</td>
</tr>
<tr>
<td>M-CSF MiaPaCa-2</td>
<td>31.3 (6.0)</td>
<td>0.149</td>
<td>65.7 (8.9)</td>
<td>0.902</td>
<td>92.1 (2.4)</td>
<td>0.620</td>
</tr>
<tr>
<td>M-CSF + IL-4 + LPS</td>
<td>9.0 (4.0)</td>
<td>0.022</td>
<td>65.2 (8.3)</td>
<td>0.710</td>
<td>99.0 (0.3)</td>
<td>0.101</td>
</tr>
<tr>
<td>M-CSF + IL-4 + LPS and MiaPaCa-2</td>
<td>16.9 (5.8)</td>
<td>0.101</td>
<td>67.2 (5.5)</td>
<td>0.805</td>
<td>86.6 (10.2)</td>
<td>0.445</td>
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<tr>
<td>M-CSF + P6</td>
<td>9.4 (2.6)</td>
<td>0.052</td>
<td>26.3 (6.3)</td>
<td>0.005</td>
<td>98.3 (0.5)</td>
<td>0.268</td>
</tr>
<tr>
<td>M-CSF + P6 and MiaPaCa-2</td>
<td>12.1 (5.2)</td>
<td>0.015</td>
<td>66.5 (11.1)</td>
<td>0.945</td>
<td>93.3 (2.8)</td>
<td>0.945</td>
</tr>
<tr>
<td>M-CSF + IL-10</td>
<td>33.9 (3.5)</td>
<td>0.082</td>
<td>66.9 (10.2)</td>
<td>0.876</td>
<td>30.2 (10.1)</td>
<td>0.003</td>
</tr>
<tr>
<td>M-CSF + IL-10 and MiaPaCa-2</td>
<td>66.9 (4.3)</td>
<td>0.004</td>
<td>93.8 (1.4)</td>
<td>0.048</td>
<td>83.8 (7.4)</td>
<td>0.432</td>
</tr>
<tr>
<td>M-CSF + IL-12</td>
<td>9.8 (1.6)</td>
<td>0.035</td>
<td>20.5 (6.2)</td>
<td>0.004</td>
<td>96.8 (1.1)</td>
<td>0.209</td>
</tr>
<tr>
<td>M-CSF + IL-12 and MiaPaCa-2</td>
<td>20.6 (3.4)</td>
<td>0.138</td>
<td>80.9 (5.5)</td>
<td>0.181</td>
<td>96.5 (0.6)</td>
<td>0.364</td>
</tr>
</tbody>
</table>
Figure 9. Macrophage characteristics (Studies II and III). A) Characteristics of differently stimulated GM-CSF (M1)-differentiated macrophages with and without MiaPaCa-2. *p<0.05 in comparing macrophages alone to correspondingly differentiated macrophages co-cultured with cancer cells. #p<0.05 in comparing differentiated to GM-CSF-only macrophages (with or without MiaPaCa-2 accordingly). Error bars show the standard error of the mean (SEM).
Figure 9. Macrophage characteristics (Studies I-III). B) Characteristics of differently stimulated M-CSF (M2)-differentiated macrophages with and without MiaPaCa-2. *p value <0.05 when comparing the macrophages alone to correspondingly differentiated macrophages co-cultured with cancer cells. #p value <0.05 when comparing the differentiated to M-CSF only macrophages (with or without MiaPaCa-2 accordingly). Error bars show the standard error of the mean (SEM).
Results

9.2 Migration of pancreatic cancer cells (Studies I-III)

Both GM-CSF-(M1) and M-CSF-(M2) -differentiated macrophages caused an increase in migration rate of pancreatic cancer cells. Hypoxic conditions elevated the migration rate of Panc-1 and ASPC, whereas it reduced the migration rate of MiaPaCa-2; no statistically significant changes occurred in the migration rate of the metastatic HPAF-II cell line. In hypoxic conditions, however, GM-CSF-differentiated macrophages caused an increased migration rate of both the metastatic cell lines (ASPC and HPAF-II) and reduced the migration rate of primary tumor cell lines (Panc-1 and MiaPaCa-2) as compared to rates in cancer-cell- and macrophage co-cultures in standard conditions (Figure 10).

Figure 10. Migration rate of pancreatic cancer cells on Matrigel and the effect of co-culturing with GM-CSF-differentiated macrophages and hypoxia (Study I). Co-culture with macrophages raised the migration rate of all of these four PDAC cell lines. In hypoxic conditions, the migration rate of primary tumor cell lines (Panc-1 and MiaPaCa-2) decreased, but it increased in metastatic cells (HPAF-II and ASPC) in co-cultures with macrophages. *p<0.001 as compared to cancer alone in normal conditions and #p<0.001 in comparing co-cultures of macrophages and cancer in normal and hypoxic conditions. Error bars show the standard error of the mean.

Additional stimulation with IL-6, IL-4+LPS, and IL-10 did not significantly alter the migration rate of MiaPaCa-2 or HPAF-2 cultured alone (p>0.05). In co-cultures with macrophages, however, there were significant changes occurred with all of these cytokines. IL-6, IL-4+LPS, IL-12, and P6 reduced cancer-cell migration rate in co-cultures as compared to co-cultures without the stimulants. In contrast, IL-10 further raised the migration of pancreatic cancer cells with macrophages, but not without them. Figures 11 and 12 shows the migration assay results of Study II and III.
Figure 11. Migration rates of pancreatic cancer cells on Matrigel in co-cultures with the macrophages polarized with GM-CSF and IL-6 or M-CSF and IL-4+LPS (Study II). Both GM-CSF- (polarization skewed towards M1) and M-CSF (polarization skewed towards M1) -differentiated macrophages caused increased migration rates of both MiaPaCa-2 and HPAF-II pancreatic cancer cell lines (p<0.001). Adding IL-6 to GM-CSF-differentiated macrophages and IL-4+LPS to M-CSF-differentiated macrophages, caused the macrophage-induced increase in the migration rate to reverse. *p<0.05 in compared to cancer cells alone. #p<0.05 in comparing co-cultures of macrophages and cancer with the added stimulus (IL-6 or IL-4+LPS) to the co-cultures with only GM-CSF or M-CSF. Error bars show the standard error of the mean.

Figure 12. The migration rate of pancreatic cancer cells on Matrigel in co-cultures with the macrophages polarized with M-CSF and IL-12, IL-10, or P6 (Study III). IL-12 reduced the migration rate of MiaPaCa-2 with and without macrophages. IL-10 raised the migration rate of MiaPaCa-2 in co-cultures with macrophages. P6 inhibited the macrophage-induced increase of the migration rate. *p<0.05 as compared to cancer cells alone. #p<0.05 in comparing the co-cultures of macrophages and cancer with the stimulus added (IL-12, IL-10, or P6) to the co-cultures with only M-CSF. Error bars show the standard error of the mean.
9.3 Changes in signaling pathways (Studies I-III)

In Study I, we examined expression of ADAM8, ADAM9, ADAM10, ADAM17, MMP2, MMP9, TIMP1, and TIMP3 with RT-PCR in the pancreatic cancer cells and compared it with the expression in pancreatic cancer cells co-cultured with macrophages. Macrophage co-culture activated expression of ADAM8 in the primary cell lines Panc-1 and MiaPaCa-2 but not in the metastatic cell lines. Co-culturing also raised the expression of ADAM10 in MiaPaCa-2 and of ADAM17 in MiaPaCa-2 and HPAF-II. Macrophages reduced in Panc-1 the expression of MMP2, which was undetectable in other cell lines. TIMP1 (an MMP2 inhibitor) remained unchanged. Co-culture with macrophages activated MMP9 expression in all cell lines and activated its inhibitor TIMP3 in all but HPAF-II. The macrophage-induced rise in migration rate and the expression of MMP9 in the different cancer cell lines showed a positive correlation ($R^2=0.93$, p=0.03).

By reducing the ADAM8 or MMP9 expression with the siRNA technique, we were able to reduce the macrophage-induced rise in migration rate in pancreatic cancer cells (MiaPaCa-2 and Panc-1, p<0.001) (Figure 13). In contrast, the migration rate of single pancreatic cancer cells with siRNA inhibition remained statistically unchanged. The relative reduction in ADAM8 and MMP9 expression by siRNA was confirmed by Western blotting.

![Graph showing migration rate in pancreatic cancer cells](image)

**Figure 13.** Decrease in ADAM8 and MMP9 expression by siRNA inhibited the macrophage-induced rise in migration rate in Panc-1 and MiaPaCa-2 (Study I). *p<0.001 as compared to the respective control. Non-targeting siRNA (NT siRNA) induced no statistically significant changes.
In Study II, we used a cytokine array panel to detect changes in 36 cytokines and chemokines and compared the effects of GM-CSF- and M-CSF-differentiated macrophages and stimulation with IL-4+LPS and IL-6 respectively in single-cultures or in co-cultures with MiaPaCa-2 cells.

In GM-CSF-differentiated macrophages (polarized more towards M1), their co-culture with pancreatic cancer cells reduced secretion of the inflammatory cytokines TNFα (from 1.35% relative to the positive control to 0.68%, p=0.018) and IL-23 (from 0.94% to 0.58%, p=0.005) and anti-inflammatory IL-13 (from 1.16% to 0.47%, p=0.010) and IL-1Ra (IL-1 receptor antagonist, from 37.13% to 2.48%, p<0.001). IL-6 in the co-cultures elevated the secretion of TNFα (to 1.35% relative to the positive control, p=0.010), IL-23 (1.08%, p=0.004), and CCL1 (chemokine ligand 1, from 1.02% to 2.41%, p=0.033) as compared to the co-cultures without IL-6.

In M-CSF-differentiated macrophages (polarized more towards M2) secretion of IL-1Ra decreased from 35.91% relative to the positive control to 16.34% (p=0.007). In the co-cultures of M-CSF-differentiated macrophages and MiaPaCa-2 the supplements IL-4+LPS raised secretion of IL-6 (from 0.75% to 4.91%, p=0.033), CCL1 (from 0.35% to 1.26%, p=0.035), CCL3 (from 1.57% to 4.15%, p=0.042), CCL4 (from 2.49% to 10.80%, p=0.027), CCL5 (from 9.66% to 27.87%, p=0.045), INFγ (from 0.69% to 1.24%, p=0.045), and TNFα (from 0.74% to 4.47%, p<0.001).

In Study III, we examined STAT 1-, 3-, 5-, AKT-, and NFkB activation by flow cytometry in M-CSF-differentiated macrophages (M2) and MiaPaCa-2 cells and stimulation/inhibition with IL-10, IL-12, and P6. The proportion of STAT 3-, STAT 5-, AKT-, and NFkB-positive macrophages increased significantly (p<0.005) in co-cultures with MiaPaCa-2 cells (Figure 14). In macrophage cultures, IL-10 raised STAT 3, 5, and NFkB, whereas in macrophage-MiaPaCa-2 co-cultures, IL-10 lowered NFkB (p<0.05). The pan-JAK/STAT inhibitor P6 reduced STAT 1 and 5, as well as AKT activity in co-cultures (p<0.05). In MiaPaCa-2 the co-culture with macrophages activated STAT 3 from 3.5% baseline activity to 0.8% ±1.0 SEM (p<0.001). This was reduced to 0.2% ±1.3 SEM (p<0.001) positivity with P6. In MiaPaCa-2, P6 also raised the NFkB activity to 11.9% ±1.8 SEM (p=0.0017) in the co-culture with macrophages. IL-10 and IL-12 made no significant changes in the intracellular signaling of MiaPaCa-2 cultured alone or in co-cultures with macrophages.
Results

Figure 14. Intracellular activation of M-CSF-differentiated macrophages (M2) in response to MiaPaCa-2 and IL-10, IL-12, and P6 measured by flow cytometry. Co-culture with MiaPaCa-2 activated STAT 1, STAT 3, STAT 5, AKT, and NFkB in macrophages. IL-10 activated STAT 3, STAT 5, and NFkB in macrophages, and IL-12 reduced STAT 1 activation and increased STAT 5. *p<0.05 as compared to macrophages cultured alone (reference was set to 3.5% activity in non-stimulated macrophages). #p<0.05 as compared to macrophages in co-culture with MiaPaCa-2 without any additional stimulation.
9.4 Prognostic significance of preoperative laboratory markers (Study IV)

The 5-year disease-specific survival was 19% (standard error 2.7%) and overall survival 17% (2.6%). Preoperatively elevated CRP and low albumin were each associated with shorter survival time in both univariate (Table 10, Figure 15) and multivariate analysis (Table 11). Moreover, the elevated tumor markers CA19-9 and CEA showed a significant association with worse outcome. CA19-9 levels associated with patient outcome both in univariate and multivariate analysis, CEA showed a significant association with patient outcome in the multivariate analysis only when categorized according to normal and elevated (≤5/>5 μg/L) values, but not when serving as a continuous variable (Table 11). The bilirubin, platelets, and leukocytes that we also determined for Study IV were not associated with survival in univariate analyses (p>0.1), and therefore they were excluded from the multivariate model.

Table 10. Univariate analysis of preoperative laboratory biomarkers' association with median survival time (months).

<table>
<thead>
<tr>
<th>Factor</th>
<th>n</th>
<th>Median survival time (months)</th>
<th>p</th>
<th>HR (95% CI)</th>
<th>p (HR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor</td>
<td>n</td>
<td>Median survival time (months)</td>
<td>p</td>
<td>HR (95% CI)</td>
<td>p (HR)</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td></td>
<td>IQR</td>
<td>HR</td>
<td>95% CI</td>
<td>p</td>
</tr>
<tr>
<td>≤5.0</td>
<td>130</td>
<td>31.8 (25.7–37.9)</td>
<td>&lt;0.001</td>
<td>1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5.1–15.0</td>
<td>58</td>
<td>26.3 (21.5–31.2)</td>
<td>1.4 (1.02–2.04)</td>
<td>0.040</td>
<td></td>
</tr>
<tr>
<td>&gt;15.0</td>
<td>42</td>
<td>14.4 (4.4–24.4)</td>
<td>2.3 (1.6–3.3)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>CA19-9 (kU/L)</td>
<td>133.5 (501.0)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤37</td>
<td>81</td>
<td>33.5 (25.9–41.1)</td>
<td>1</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>38–554</td>
<td>114</td>
<td>26.0 (20.0–32.0)</td>
<td>1.5 (1.01–2.0)</td>
<td>0.022</td>
<td></td>
</tr>
<tr>
<td>≥555</td>
<td>60</td>
<td>16.1 (7.4–24.7)</td>
<td>2.1 (1.5–3.1)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>CEA (μg/L)</td>
<td>2.7 (2.7)</td>
<td></td>
<td></td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>≤5.0</td>
<td>205</td>
<td>27.4 (23.7-31.1)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;5.0</td>
<td>47</td>
<td>14.4 (22.7-30.0)</td>
<td>1.5 (1.1-2.2)</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>37.0 (4.3)*</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>≤30.0</td>
<td>16</td>
<td>10.0 (2.9-17.0)</td>
<td>3.0 (1.8-5.0)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>30.1–35.9</td>
<td>83</td>
<td>18.1 (13.1-23.0)</td>
<td>1.6 (1.2-2.2)</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>≥36.0</td>
<td>160</td>
<td>31.7 (26.6-36.8)</td>
<td>1</td>
<td></td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

IQR, interquartile range; HR, hazard ratio; CI, confidence interval; CRP, C-reactive protein; CEA, carcinoembryonic antigen
* mean albumin (standard deviation)
Results

Figure 15. Survival curves. The effect of CRP, albumin, CA19-9, and CEA on survival of the patients resected for pancreatic ductal adenocarcinoma. Survival curves determined by Kaplan-Meier analysis, significances by log-rank test.
The association of CRP, albumin, CA19-9, and CEA with survival time showed that the higher the median preoperative CRP, CA19-9, and CEA, and the lower the mean albumin, the shorter was the survival time (Figure 16). Besides CRP, albumin, and the tumor markers, in the multivariate model, adjuvant chemotherapy, margin involvement, and lymph-node ratio (LNR) showed a significant association with survival (Table 11). Patients who died of PDAC had higher median CRP and CA19-9 and lower albumin levels than did those alive throughout follow-up.

Table 11. Multivariate model considering clinically and statistically important patient characteristics and preoperative biomarkers (n=189).

<table>
<thead>
<tr>
<th>Factor</th>
<th>Description</th>
<th>HR (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>&lt;65/≥65</td>
<td>0.97 (0.68-1.38)</td>
<td>0.866</td>
</tr>
<tr>
<td>Sex</td>
<td>Male/Female</td>
<td>1.27 (0.90-1.79)</td>
<td>0.173</td>
</tr>
<tr>
<td>Tumor size (mm)</td>
<td>≤30/&gt;30</td>
<td>1.02 (0.71-1.48)</td>
<td>0.906</td>
</tr>
<tr>
<td>T status</td>
<td>T1-2/T3-4</td>
<td>1.32 (0.90-1.93)</td>
<td>0.16</td>
</tr>
<tr>
<td>Adjuvant therapy</td>
<td>Yes/No</td>
<td>1.86 (1.29-2.68)</td>
<td>0.001</td>
</tr>
<tr>
<td>Margin</td>
<td>R0/R1</td>
<td>1.77 (1.15-2.72)</td>
<td>0.009</td>
</tr>
<tr>
<td>LNR (0)</td>
<td>N0</td>
<td>1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LNR</td>
<td>N1, ≤0.2</td>
<td>1.83 (1.22-2.75)</td>
<td>0.004</td>
</tr>
<tr>
<td>LNR</td>
<td>N1, &gt;0.2</td>
<td>3.64 (2.08-6.38)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>log</td>
<td>0.18 (0.05-0.75)</td>
<td>0.019</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>log</td>
<td>1.20 (1.03-1.40)</td>
<td>0.021</td>
</tr>
<tr>
<td>CEA (μg/L)</td>
<td>log</td>
<td>1.20 (0.93-1.56)</td>
<td>0.168</td>
</tr>
<tr>
<td>CA19-9 (kU/L)</td>
<td>log</td>
<td>1.11 (1.02-1.21)</td>
<td>0.014</td>
</tr>
</tbody>
</table>

HR, hazard ratio; CI, confidence interval; LNR, lymph-node ratio
Results

Figure 16. Laboratory data according to survival time. Association of median CRP, mean albumin, median CA19-9, and median CEA with survival time categorized as less than 3 months, 3-12 months, 12-24 months, and over 24 months. Patients with shorter postoperative survival time had higher preoperative median CRP, CA19-9, and CEA, and lower mean albumin level. Significances determined by the Jonckheere-Therpstra test.
GPS and mGPS both associated in univariate analysis with survival time of patients with PDAC (Table 12, Figure 17). In multivariate analysis considering age, sex, tumor size, T status, administration of adjuvant therapy, margin involvement, LNR, CA19-9, and CEA (Table 11), both GPS score of 1 and mGPS score of 1 had no statistical significance regarding survival (Table 12). Hazard ratios for both GPS 2 and mGPS 2 were each 2.1, indicating that neither seemed clearly superior for our study patients in predicting their outcome.

**Table 12.** The median survival time categorized by the GPS and mGPS. The hazard ratios (HR) by the Cox regression model in univariate analysis and in the multivariate model (taking into account age, sex, tumor size, adjuvant therapy, T-status, lymph-node ratio, CA19-9, and CEA).

<table>
<thead>
<tr>
<th>Factor</th>
<th>Median survival time (95% CI)</th>
<th>p (survival time)</th>
<th>Univariate model</th>
<th>Multivariate model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>time</td>
<td>HR (95% CI)</td>
<td>p (HR)</td>
</tr>
<tr>
<td>GPS</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>67</td>
<td>21.5 (14.6-28.3)</td>
<td>1.6 (1.1-2.2)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>33</td>
<td>14.4 (4.3-24.6)</td>
<td>2.3 (1.5-3.4)</td>
</tr>
<tr>
<td>mGPS</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0</td>
<td>194</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>26</td>
<td>20.7 (7.3-34.1)</td>
<td>1.8 (1.1-2.8)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>33</td>
<td>14.4 (4.3-24.6)</td>
<td>2.1 (1.5-3.1)</td>
</tr>
</tbody>
</table>

**Figure 17. Survival curves by GPS.** Association of Glasgow prognostic score (GPS) and modified Glasgow prognostic score (mGPS) on survival of patients with resected pancreatic ductal adenocarcinoma. Survival curves (survival time in months after surgery) by Kaplan-Meier analysis and significance by log-rank test.
10 DISCUSSION

These studies provide novel insight into the role of inflammation and especially macrophages in pancreatic ductal adenocarcinoma (PDAC). The significant findings include our being able through macrophage polarization to modify the effect of those macrophages on PDAC cell migration. We showed that macrophages activate pancreatic cancer cell migration by causing increases in MMP9 and ADAM8 expression in PDAC cells, and we showed that supporting the inflammatory microenvironment in PDAC and macrophage co-cultures had an inhibitory effect on PDAC cell migration. A relevant finding was also preoperative SIR’s adverse effect on the prognosis of resectable PDAC.

Macrophages can, due to their plasticity, change their phenotype and characteristics in response to stimuli in their microenvironment (Rhee 2016). We observed their polarization by measuring the surface expression of several surface-protein markers. First, the isolated monocytes must mature into macrophages, a process which we induced with either GM-CSF or M-CSF. The original hypothesis was that GM-CSF would polarize the macrophages into type M1 (inflammatory) and M-CSF into type M2 (anti-inflammatory) macrophages (Hamilton 2008, Jaguin 2013). However, our study demonstrated that with these growth factors the polarization was incomplete; GM-CSF and M-CSF as such were insufficient to skew the macrophages to the two extremes of macrophage polarization. Further, both macrophage lines promoted cancer cell migration, indicating that PDAC cells skewed the GM-CSF-differentiated M1 macrophages into type M2 TAMs. Co-culturing the macrophages with MiaPaCa-2 cancer cells induced surprisingly few changes in macrophage surface expression. In theory, cancer cells should polarize the macrophages towards a subtype of M2, and raise the proportion of cells positive to the M2/TAM surface markers CD14, CD163, CD206, and CD209, while reducing the M1 markers. Instead, after addition of MiaPaCa-2 to GM-CSF-differentiated macrophages, only the CD16 (M1 marker) decreased; and in M-CSF-differentiated macrophages, no statistically significant changes occurred. We were able to change the surface expression of the macrophages with added cytokines, even though polarization indicated by the surface markers was somewhat inconsistent with the additional stimuli. It seemed, though, that scavenger receptor CD163, widely used to differentiate M2 from M1, responded as expected to the polarization stimuli.

In the light of our and numerous others’ results, it seems evident that macrophages play a critical role in the regulation and modulation of cancer cell migration, which is a key step leading to cancer metastasis (Beuran 2015).
Our results show that macrophages can either resist or activate cancer cell migration according to the stimuli of their microenvironment. That the macrophages’ effect can be altered by changes in the surrounding cytokine profile, even after their maturation, is exciting. This raises the question whether modifying a patient’s macrophage polarization could provide a suitable target for anti-cancer therapy.

The inflammatory cytokines IL-6 and IL-12, typically produced by M1 macrophages, restricted pancreatic cancer cell migration in co-cultures with macrophages, and thus seemed to promote the anti-tumor actions of M1 macrophages (Baay 2011). Conversely, IL-6 is a known activator of STAT 3, which in turn promotes cancer-cell proliferation and inhibits cancer-cell apoptosis (Yu 2009); the inhibition of pancreatic cancer-cell migration in our study was thus somewhat surprising. The cytokines IL-6, IL-10, and IL-4+LPS, without the macrophages, left the migration of pancreatic cancer cells unchanged. IL-10, however, clearly elevated the migration rate of cancer cells only in co-cultures with macrophages, indicating that something changes in the macrophages that supports the migration of pancreatic cancer cells.

When comparing the effects of macrophages on cancer-cell migration rate, it seemed that the metastatic cell lines were able to take more advantage of the macrophages than did the primary-tumor PDAC cell lines. Hypoxia, together with macrophages, caused significantly increased migration of metastatic cell lines, but not of primary-tumor cell lines. In co-cultures with macrophages, the migration rate of metastatic cell lines increased more than did the rate of primary-tumor cell lines. Further, the pro-inflammatory microenvironment (with IL-6 and IL-4+LPS) reversed the macrophage-induced increase in PDAC cell migration rate more effectively in the primary cell lines. The response to the additional stimuli varied among different cancer cell lines. Study I showed that for example the expression of ADAM8 was associated with migration rate particularly in primary-tumor cell lines, but not as closely in metastatic cell lines. The differing response of the PDAC cell lines, shown also earlier studies (Shen 2013), may reflect the heterogeneity of the disease and highlight the importance of understanding the underlying mechanisms.

The signaling pathways implicated in PDAC cells’ interactions with macrophages are complex and dependent on many factors. In our studies we focused on ADAMs, MMPs, intercellular cytokines associated with inflammation, and intracellular STAT and NFkB signaling. Significant changes occurred in all of these signaling pathways. We found that macrophages raised the migration rate of pancreatic cancer cells by activating their expression of ADAM8 and MMP9, which participate in ECM remodeling and angiogenesis (Page-McCaw 2007). Hypoxia also led to increased MMP9 expression but simultaneously induced the expression of its inhibitor TIMP3; it seemed that
their balance was a critical factor in the migration rate of each cancer cell line. Depending on cancer type, MMP9 correlates in previous studies with both better and worse prognosis and also depending on whether MMP9 was determined from peripheral blood circulation or from the tumor sample (Scorilas 2001, Grutzmann 2004, Mroczko 2009, Koskensalo 2012). It seems that while locally in the tumor microenvironment the ECM-degrading MMP9 aids tumor metastasis, it may systemically support inflammatory activation and thus play a protective role against cancer progression. Levels of MMPs and TIMPs in the blood circulation increase also in other inflammatory conditions, and they correlate with the severity of such diseases as acute pancreatitis (Nukariainen 2016).

Cytokine determination in the co-cultures of macrophages and pancreatic cancer cells showed that cytokine support of the pro-inflammatory microenvironment inhibited the macrophage-induced increase in PDAC-cell migration rate. MiaPaCa-2 cells were able to suppress the inflammatory cytokines secreted by the GM-CSF-differentiated macrophages. However, with addition of IL-6, inflammatory cytokine production was restored in the co-cultures, and the macrophage-induced rise in PDAC cell migration was inhibited. The same was observable in the M-CSF-differentiated macrophages; they had relatively low inflammatory cytokine production to begin with, and in co-cultures the cancer-cell migration rate was higher, but when we added IL-4+LPS to the co-cultures, inflammatory cytokine production was enhanced, and pancreatic cancer cell migration was inhibited. These results support the theory of the anti-tumor actions of M1 and pro-tumor actions of M2 macrophages. This indicates that the inflammatory microenvironment and the cytokines that the macrophages produce may be more important determinants of macrophage polarization than are the surface markers they express, as also shown elsewhere (Helm 2014).

In co-cultures with macrophages, P6, IL-6, and IL-4+LPS promoted TNFα secretion, and each of these also inhibited PDAC migration. In contrast, TNFα and NFKB secretion was reduced in the co-cultures supplemented with IL-10 that promoted cancer-cell migration. TNFα is an immunomodulating cytokine that regulates apoptosis, growth-factor synthesis, and participates in inflammatory- and immune responses, having both tumorigenic and anti-tumor functions. In some murine models, it causes hemorrhagic necrosis in tumors, but its systemic use in cancer treatment is limited by its ineffectiveness and severe toxicity (Balkwill 2009). Further, the constitutive production of TNFα by tumor cells has tumor-promoting actions possibly by its participating in epithelial-to-mesenchymal transition and by recruiting anti-inflammatory TAMs to the tumor site (Li 2009, Helm 2014).
Intracellular STAT transcription factors participate in tumor progression and macrophage differentiation through phosphorylation by JAK-receptors. In macrophages, the co-culture with pancreatic cancer cells activated STAT 3 and 5, both implicated in cancer progression, as were NFkB and AKT, important mediators of the immune system. Further, in MiaPaCa-2, STAT 3 was increased in the co-culture with macrophages. STAT 3 suppresses the expression of NFkB target genes and thus suppresses inflammatory activation in cells (Yu 2009), and it has earlier been associated with PDAC invasiveness (Nagathihalli 2016). P6 reduced STAT 3 in MiaPaCa-2 (but not significantly in macrophages), which may lead to inhibited cancer-cell migration. However, although IL-12 inhibited pancreatic cancer-cell migration in co-cultures, STAT 3 activity remained intact, indicating that migration rate can change despite STAT 3.

Study IV showed the connection between PDAC and inflammation also in vivo in humans, because CRP and hypoalbuminemia independently associated strongly with PDAC patients’ post-operative survival. Additionally, the serum tumor markers CA19-9 and CEA, which correlated with tumor size and T status—thus possibly reflecting an advancing stage of the disease—were associated with outcome. Tumor markers are associated with advancing stage of cancer, and their fluctuations correlate with response to treatment and disease progression (Goonetilleke 2007). In contrast, elevated CRP had no significant association with the tumor-related factors that typically associate with the prognosis (tumor size, T status, LNR). This may indicate that SIR is activated independent of these factors. What remains uncertain, however, is whether SIR is responsible for the worse prognosis or whether it is only an indicator of a more aggressive cancer.

Our multivariate analysis showed that, besides CRP, albumin, CA19-9, and CEA, factors associated with survival were LNR, resection margin status, and adjuvant chemotherapy. These factors, however, have the obvious prognostic disadvantage of emerging only during or after surgery. This means that they are undeterminable in planning of the surgery and in evaluation of post-operational prognosis. Therefore, determination of preoperative CRP, combined with albumin and tumor markers, may prove helpful for selecting patients for surgery, although further validation is vital. The International Study Group of Pancreatic Surgery (ISGPS) has also acknowledged the importance of SIR in PDAC in their recent guidelines that recommend, in all patients considered for resection of PDAC, determination of either mGPS or the NLR (Bockhorn 2014).

Although the present study provides novel insight into the complex field of PDAC research, some noteworthy issues remain. First, increased migration and invasion in the ECM indeed is the preliminary step in the metastasis
cascade, but pancreatic cancer progression comprises much more than migration. In our experiments, we ignored, however, apoptosis, cell viability, and proliferation and these would provide further understanding of PDAC progression. In the co-culture assays with pancreatic cancer cells and macrophages, where we measured the secretion of cytokines in the culture medium, it was, unfortunately, undeterminable whether the increased secretion came from the TAMs or from cancer cells. Further, the problem with the inhibition of migration rate is that we cannot be sure whether the stimuli actually affected migration rate through the macrophages; it is also possible that the stimuli themselves hinder the pancreatic cancer cells’ raising of the migration rate in response macrophage signals. What was evident, however, was that IL-10 alone did not change the cancer-cell migration rate, but in co-cultures with macrophages it clearly changed the macrophage phenotype toward an anti-inflammatory direction, which led to increased pancreatic cancer-cell migration. In the signaling-pathway assays (assessing ADAMs, MMPs, and STATs), we were able to determine whether the pathway was activated in pancreatic cancer cells or in macrophages.

Finally, because all cell studies were conducted in \textit{in vitro} cell cultures and with commercial PDAC cell lines, it is impossible to draw direct conclusions regarding \textit{in vivo} settings. The validation of our results in non-commercial cancer cells and \textit{in vivo} settings is a field for future research. In contrast, the macrophages in our studies came directly from healthy human donors, and were not immortalized or otherwise genetically modified. Many studies in this field uses commercial murine macrophages. It is exceedingly important to create basic knowledge in reproducible settings of the mechanisms underlying PDAC progression, because the treatment possibilities against metastasis are currently inadequate; hope exists for finding novel targets for therapy, possibly by changing the interaction between PDAC and TAMs. Nywening \textit{et al.} (2016) recently showed very promising results in a phase 1 trial by inhibiting CCR2, which recruits TAMs and enhances immune-suppression in PDAC, in combination with FOLFIRINOX. This further encourages exploration of the possibilities of TAM-targeting therapies.

Study IV is, to our knowledge, one of the largest studies concerning the association of resectable PDAC with the prognostic value of SIR. Yet, because of some missing data among laboratory values and pathologic specimens, the final multivariate analyses comprised only 189 patients as compared to the 265 originally included. A larger cohort would provide greater reliability, but our comprehensive and reliable clinical data enhance the value of the study. What we did not determine was the duration of the symptoms prior to diagnosis, data which may correlate with PDAC prognosis (Porta 2005). Symptom duration may be associated with development of laboratory abnormalities (Watanabe 2004) and with survival, as duration may indicate longer disease
progression. However, the symptoms of pancreatic cancer typically occur as the disease progresses, with symptoms of early disease often being vague (Hidalgo 2010, Ryan 2014). This makes it difficult to retrospectively determine the onset of symptoms, especially in resectable PDAC; for example, back pain predicts inoperable disease (Ridder 1995).

For the future, Study IV, in concert with the other studies, raises the question whether patients with preoperative SIR may benefit from treatment with anti-inflammatory drugs or neoadjuvant therapy. Further, what remains uncertain is whether SIR is a cause or only an indicator of worse outcome; it would be interesting to study whether SIR is associated with the pre-diagnostic duration of symptoms, and if it predicts metastatic disease not yet visible by imaging, or predicts earlier progression of PDAC.

Between 1981 and 2010, the overall 1-year relative survival rate for pancreatic cancer has risen from 3% to 7%, and 5-year relative survival from 17% to 28% (Sun 2014), indicating slow positive progress in the management of this frightening disease. With the rising incidence of pancreatic cancer, it is now more important than ever to create novel strategies for PDAC management. The introduction of new treatments has been slow, with radical resection remaining the only possibility for a cure. Yet, some light glimmers at the end of the tunnel, as cancer research rapidly progresses. Our studies encourage further exploration of the possibilities of targeting inflammatory processes and macrophages as possible candidates for anti-cancer therapy.
11 CONCLUSIONS

The studies included in this thesis brought novel insight to the association of pancreatic cancer and inflammation and especially the interaction of pancreatic cancer cells and tumor-associated macrophages. Macrophages participate centrally in the regulation and promotion of pancreatic cancer-cell migration and are potential targets for therapy. Based on these studies, we conclude the following:

1. Hypoxia, MMP9, and ADAM8 have an essential effect on the macrophage-induced increase in pancreatic cancer cell migration.

2. Macrophages can be polarized by various stimuli towards types M1 and M2 macrophages, but their surface protein expression is not always directly indicative of their impact on pancreatic cancer cell migration; however, it seems that the more inflammatory the microenvironment, the more effectively the macrophages inhibit cancer cell migration.

3. Pancreatic cancer cell migration can be both promoted and inhibited by stimulating the macrophages with differing cytokines.

4. In macrophages, their STAT 1, 3, and 5, as well as NFkB/Akt pathways were activated by their interaction with PDAC cells. The pan-JAK/STAT inhibitor P6 inhibited PDAC cell migration.

5. In patients with resectable pancreatic ductal adenocarcinoma, a preoperative systemic inflammatory response predicts worse prognosis.
12 ACKNOWLEDGEMENTS

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