Joni Leppänen

THE ROLE OF HYPOXIA, INNATE IMMUNITY RECEPTORS AND STROMAL RESPONSE IN PANCREATIC CANCER
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University of Oulu, P.O. Box 8000, FI-90014 University of Oulu, Finland

Abstract
Pancreatic cancer remains one of the deadliest malignancies, with dismal prognosis. Pancreatic cancer arises from precursor lesions called pancreatic intraepithelial neoplasia. Toll-like receptors (TLR) are receptors of the innate immunity responsible for initiating immune responses against invading pathogens. Their involvement in cancer progression is becoming evident. Hypoxia is a typical characteristic of pancreatic cancer and linked to poor prognosis. Typically, pancreatic cancer has an abundant desmoplastic stroma that contributes to the hypoxia and poor delivery of anti-tumor drugs to the cancer cells. Tenascin-C and fibronectin are proteins of the extracellular matrix. They are involved in normal organ development, but in recent years, their involvement in various cancers has become evident.

This thesis examined the involvement of Toll-like receptors, hypoxia markers HIF-1alpha and Carbonic anhydrase 9 (CAIX) as well as stromal markers tenascin-C and fibronectin in pancreatic cancer. Furthermore, the prognostic effect of each protein was evaluated. The material consisted of whole section tissue samples of surgically treated patients with pancreatic ductal adenocarcinoma. The expression of the proteins was evaluated using immunohistochemical stainings. TLR, HIF-1alpha, CAIX, tenascin-C and fibronectin were all abundantly expressed in pancreatic cancer. High TLR9 associated with improved prognosis while weak HIF-1alpha indicated poor prognosis. In a subgroup analysis consisting of only T1 and T2 tumors, high tenascin-C associated with poor prognosis. There was no significant correlation between TLR and hypoxia marker expression.

Based on these results, TLR2, TLR4 and TLR9 are expressed in pancreatic cancer, and high TLR9 associates with improved survival of the patients. Weak HIF-1alpha associated with poor prognosis, suggesting that other factors than hypoxia might be involved in the regulation of HIF-1alpha expression. Tenascin-C and fibronectin are not associated with patient prognosis in pancreatic cancer.

Keywords: hypoxia, innate immunity, microenvironment, pancreatic cancer, pancreatic intraepithelial neoplasia, stroma, Toll-like receptor


Tulosten perusteella haimasyövällä on runsaasti Tollin kaltaisia reseptoreita, ja korkea TLR9 on yhteydessä parantuneeseen ennusteeseen. Matala HIF-1alpha on yhteydessä huonoon ennusteeseen. Tenaskiinilla ja fibronektiiniillä ei ole vaikutusta potilaiden ennusteeseen.

Asiasanat: haiman tiehyidenisäinen neoplasia, haimasyöpä, hypoksia, luontainen immuniteetti, mikroympäristö, sidekudos, Tollin kaltainen reseptori
Acknowledgements

This study was carried out at the University of Oulu Cancer and Translational Medicine Research Unit, Department of Pathology, Department of Surgery and Medical Research Center Oulu. I wish to acknowledge the financial support provided by The Finnish Medical Foundation, Finnish Cultural Foundation, Emil Aaltonen Foundation, Päivikki and Sakari Sohlberg Foundation and Mary and Georg C Ehrnroot Foundation.

I am deeply grateful for the guidance and supervision given by Professor Tuomo Karttunen, docent Juha Saarnio and Professor Petri Lehenkari. Tuomo has had a key role in the progress of this study, and without him this study would not have been possible. His encouraging attitude and never-ending ideas combined with his vast experience have kept my motivation up through these years. I also want to thank Tuomo for providing the facilities that made this research possible. I want to thank Juha Saarnio for offering me this amazing opportunity to begin my scientific career. I value his encouraging words and guidance in research as well as in life in general. Our coffee breaks and meetings have given me perspective into research, but most of all, into life as a medical doctor. When things have not gone as planned, Petri Lehenkari has been the man of the hour. I want to thank him for providing me with excellent ideas to improve my research. His never-ending ideas have kept the flame of motivation burning. It has been a pleasure to work with these gentlemen.

I want to thank the Center of Excellence for providing an outstanding atmosphere to do scientific research. I value highly the hands-on guidance provided by Dr Olli Helminen and Dr Heikki Huhta; their help and enthusiasm towards scientific research has made this work possible. I wish to thank Docent Joonas Kauppila for endless ideas and encouragement even in the hardest of times. Without these excellent gentlemen this work would not have been possible, and I am looking forward to continue working with these fine men in the future. All I can say is “mielettömiä miehiä”.

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The life of a researcher and medical doctor can sometimes be tough, and therefore recreational time is vital. I want to thank all my friends, especially Ville Lindholm, Okke Nikkinen, and the rest of the “Isännistö” for giving me the possibility to cool of my brain. I think the moments spent with you guys have been one of the finest moments of my life so far. I also want to thank the men of “McMurray” for intriguing conversations, and Tuomas Laukka and Jani Luukkonen for sharing the challenges of researchers’ life as well as the moments spent in the mountains. I want to thank my family for the support they have given me in my life. Finally, I want to thank my dearest Lotta for the love and support she has given me in these years. I am looking forward to our adventures in the future.

1.1.2019

Joni Leppänen
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
</tr>
<tr>
<td>CAIX</td>
<td>Carbonic anhydrase 9</td>
</tr>
<tr>
<td>CA19-9</td>
<td>Carbohydrate antigen 19-9</td>
</tr>
<tr>
<td>CCL3</td>
<td>Chemokine ligand 3</td>
</tr>
<tr>
<td>CCL11</td>
<td>Chemokine ligand 11</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>CLR</td>
<td>C-type lectin receptor</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography Scan</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>FAMM</td>
<td>Familial atypical multiple mole melanoma</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis</td>
</tr>
<tr>
<td>FPC</td>
<td>Familial pancreatic cancer</td>
</tr>
<tr>
<td>HIF-1alpha</td>
<td>Hypoxia-inducible factor 1 alpha</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IL12</td>
<td>Interleukin 12</td>
</tr>
<tr>
<td>IRAK</td>
<td>Interleukin-1 receptor-associated kinase</td>
</tr>
<tr>
<td>IRF3</td>
<td>Interferon regulatory factor 3</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Matrix metalloproteinase-9</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response 88</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK-cells</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>TAK1</td>
<td>Transforming growth factor beta-activated kinase</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TME</td>
<td>Tumor microenvironment</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>Tp53</td>
<td>Tumor protein 53</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor associated factors</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon-β</td>
</tr>
<tr>
<td>TSR</td>
<td>Tumor-stroma ratio</td>
</tr>
<tr>
<td>PanIN</td>
<td>Pancreatic intraepithelial neoplasia</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PDAC</td>
<td>Pancreatic ductal adenocarcinoma</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern-recognition receptor</td>
</tr>
<tr>
<td>PSC</td>
<td>Pancreatic stellate cells</td>
</tr>
<tr>
<td>REG4</td>
<td>Regenerating islet-derived protein 4</td>
</tr>
<tr>
<td>RIP1</td>
<td>Receptor-interacting protein 1</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-I-like receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RS-virus</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
List of original publications

This thesis is based on the following publications, which are referred to throughout the text by their Roman numerals:


*Equal contribution*
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Tiivistelmä
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2.2 Innate immunity

2.3 Hypoxia

2.4 TLR and hypoxia interplay
1 Introduction

Pancreatic cancer is one of the deadliest malignancies in the gastrointestinal tract. Typically, pancreatic cancer is detected late as symptoms such as abdominal pain, jaundice and weight loss occur. At this stage, pancreatic cancer has often spread from the pancreas to lymph nodes, nearby organs and blood vessels, and sent metastases to the liver and other organs. This late stage makes the tumor inoperable, and therefore only some of the tumors can be treated with surgery. Even with surgery, the survival rates remain extremely low, as it is typical for pancreatic cancer to relapse, and even at the time of surgery, microscopically small metastases may be present, undetectable with modern imaging methods. The current 5-year survival rates are only around 5-15%, with little variance seen between different regions. In the latest 20 years, only minor improvement in survival rates is seen. The surgery itself is usually very demanding for the patient, as it involves removing large portions of the pancreas as well as other organs such as the gall bladder, parts of the small intestine, and sometimes, the spleen.

Typical characteristics of pancreatic cancer are abundant dense stroma around the tumor islets and hypovascularization. These contribute to resistance against many chemotherapeutic drugs, as the delivery of these drugs is impaired by the dense stroma and loose net of blood vessels around the cancerous cells. These make treatment of pancreatic cancer challenging. Pancreatic cancer arises from local microscopic precursor lesions called pancreatic intraepithelial neoplasia (PanIN). PanINs contain features of pancreatic cancer, but do not invade through the basal membrane.

Toll-like receptors (TLR) are receptors of innate immunity, the first line of defense against pathogens coming from outside the organism. TLRs can be found on the surface of immune cells as well as on the epithelium of various organs. Furthermore, their expression can be found in many solid tumors. Each TLR has their specific ligands that activate signaling cascade ultimately leading to immune response against invading pathogens. In addition to pathogens, TLR also recognize various damage-associated proteins of the host. In recent years, knowledge of their presence in various malignancies has increased and evidence of their involvement in patient prognosis in many cancers is ever more plentiful.

Lack of oxygen leads to hypoxia in healthy and diseased organism. Hypoxia-inducible factor 1 alpha (HIF-1alpha) and carbonic anhydrase 9 (CAIX) are proteins expressed under hypoxic conditions. Hypoxia contributes to selection of cancer cells that are more aggressive and resistant to lack of oxygen. Previously,
high levels of HIF-1alpha and CAIX have been linked to adverse prognosis in many cancer types. Furthermore, a link between hypoxia and innate immunity and inflammation activation in cancer has been suggested.

The tumor microenvironment consists of fibroblasts, immune and inflammatory cells, lymphocytes, blood vessels and the extracellular matrix. In recent years, its significance in cancer progression has become evident. Tumor microenvironment is in constant interaction with the cancer cells. Tenascin-C and fibronectin are glycoproteins of the extracellular matrix, and their presence and involvement in patient survival in various malignancies have been suggested. Furthermore, preliminary evidence suggests interplay between tenascin-C and TLRs in the formation of inflammatory stroma.

The aim of this study was to evaluate TLR, HIF-1alpha and CAIX, as well as tenascin-C and fibronectin expression on pancreatic cancer and investigate their impact on patient prognosis. In addition, TLR, HIF-1alpha and CAIX expression was evaluated in pancreatic ductal intraepithelial neoplasia.
2  Review of the literature

2.1 Pancreatic cancer

Pancreatic cancer has very poor prognosis worldwide (Kamisawa, Wood, Itoi, & Takaori, 2016a). Over 90% of malignant pancreatic tumors are pancreatic ductal adenocarcinomas (Hidalgo et al., 2015). Other types of malignant pancreatic tumors include pancreatic neuroendocrine tumors, which comprise around 5% of malignant pancreatic tumors. Rare types of malignant pancreatic tumors comprising only 1-2% of malignant tumors each, include acinar cell carcinoma, solid pseudopapillary neoplasms and pancreatoblastoma (Hackeng, Hruban, Offerhaus, & Brosens, 2016) (Table 1). Typically, the majority of the patients remain symptom-free until the advanced stage of the disease, making it harder to detect the malignancy at an early stage (Falasca, Kim, & Casari, 2016).

Table 1. Prevalence of malignant pancreatic tumors (Hackeng, Hruban, Offerhaus & Brosens 2016)

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Prevalence</th>
<th>Mean age at diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic ductal adenocarcinoma</td>
<td>90%</td>
<td>66</td>
</tr>
<tr>
<td>Pancreatic neuroendocrine tumor</td>
<td>5%</td>
<td>58</td>
</tr>
<tr>
<td>Solid pseudopapillary neoplasm</td>
<td>1-2%</td>
<td>29</td>
</tr>
<tr>
<td>Acinar cell carcinoma</td>
<td>1-2%</td>
<td>56</td>
</tr>
<tr>
<td>Pancreatoblastoma</td>
<td>&lt;1%</td>
<td>5</td>
</tr>
</tbody>
</table>

Symptoms caused by pancreatic cancer include jaundice, abdominal pain, weight loss and light-colored stools. Only around 15-20% of the patients are eligible for surgical treatment; the rest are diagnosed with metastatic or locally spread disease, unsuitable for curative surgery (Khorana et al., 2016; Zhan, Xu, Wu, Zhang, & Hu, 2015). Approximately 5-10% of patients have borderline resectable disease, a condition where anatomical and biological factors must be addressed to determine the operability of the tumor (Isaji et al., 2018). In the recent years, piling evidence has shown promising results of combining neoadjuvant therapy with surgical intervention in locally advanced pancreatic cancer and borderline resectable disease, however, more evidence is needed to establish surgical treatment for locally advanced pancreatic cancer and borderline resectable disease (Kadera et al., 2014; Kommalapati, Tella, Goyal, Ma, & Mahipal, 2018). Even with surgical treatment, the 5-year survival rates remain extremely poor (Ilic & Ilic, 2016).


2.1.1 Risk factors

Several risk factors have been identified to increase the risk of pancreatic cancer (Wormann & Algul, 2013). Age appears to be an important risk factor for pancreatic cancer. Pancreatic cancer rarely develops at a young age, and the majority of the patients are diagnosed at the age of 40–80, the median age at diagnosis being 71 years (Bosetti et al., 2012). There is a difference in incidence between the sexes: pancreatic cancer appears to be approximately 30% more common in males (Midha, Chawla, & Garg, 2016). Smoking and alcohol abuse are known risk factors for developing pancreatic cancer, as is chronic pancreatitis, which is most commonly caused by excessive alcohol usage (Ezzati, Henley, Lopez, & Thun, 2005; Lowenfels et al., 1993). Patients with chronic pancreatitis have up to 13 to 16 times higher risk for developing pancreatic cancer (Kirkegard, Mortensen, & Cronin-Fenton, 2017; Raimondi, Lowenfels, Morselli-Labate, Maisonneuve, & Pezzilli, 2010).

Obesity and nutritional factors, such as high consumption of meat, cholesterol and foods containing high levels of nitrosamines, have also been identified to increase the risk of developing pancreatic cancer (Appleby, Crowe, Bradbury, Travis, & Key, 2016; Aune et al., 2012; Zheng & Lee, 2009). Diabetes is also identified as a risk factor for pancreatic cancer, however, it may also be a symptom of pancreatic cancer (Maisonneuve & Lowenfels, 2015).

The majority of the patients develop pancreatic cancer through sporadic mutations, but also hereditary conditions explain a small proportion of pancreatic cancers. It has been estimated that approximately 10% of all pancreatic cancers may be hereditary (Salo-Mullen et al., 2015).

BRCA1 and BRCA2 mutations occur in approximately 1/550 individuals, even though some variance is seen depending on the population (Paluch-Shimon et al., 2016). BRCA1 and BRCA2 is associated with ovarian and breast cancer, but also is a risk factor for pancreatic cancer (Leung & Saif, 2013).

Peutz-Jeghers syndrome is a rare syndrome, associated with mucocutaneous pigmentation and intestinal hamartomatous polyps (Jelsig et al., 2014). Peutz-Jeghers syndrome is a risk factor for colon, stomach, small intestine, breast and pancreatic cancer (Resta et al., 2013).

Familial Adenomatous Polyposis (FAP) is a syndrome characterized by multiple adenomas in the colon and polyps in the gastric fundus and duodenum. The risk for developing colorectal carcinoma is nearly 100% (Waller, Findeis, &
Lee, 2016). FAP poses a risk for pancreatic cancer, the risk being around 4.5 higher than in general population (Moussata et al., 2015).

Lynch syndrome or hereditary nonpolyposis colorectal cancer is the most frequent cause for hereditary colorectal cancer (Lynch et al., 2009). Furthermore, the risk for developing pancreatic cancer in patients with Lynch syndrome is nearly 9 times higher than in general population (Kastrinos et al., 2009).

Li-Fraumeni syndrome is a condition characterized by wide range of malignant cancers at very young age (Correa, 2016). It has been estimated that nearly half of the individuals with Li-Fraumeni syndrome develop cancer by the age of 30. Individual with Li-Fraumeni syndrome have around 7 times higher risk for developing pancreatic cancer than general population (Ruijs et al., 2010).

### 2.1.2 Incidence

The incidence of pancreatic cancer varies between countries and continents (Ilic & Ilic, 2016). In recent years, the highest incidence rates have been seen in Northern America and Western Europe. In Western Europe, the incidence of pancreatic cancer was 7.3 per 100,000 people in 2012 (Ferlay et al., 2015). According to the Finnish Cancer Registry, there were 5,542 new pancreatic cancer cases between the years 2011 and 2015 in Finland, and the age-adjusted incidence per 100,000 people was 20.89 for both sexes.

Between 2011 and 2015, females had a slightly higher incidence with 2,883 new cases compared to 2,659 new cases in males. Between years 1956-1961 there were 728 new cases in male and 602 in female, and in years 1987-1991 the same numbers were 1,557 and 1,967, so in Finland, the incidence of pancreatic cancer has been rising steadily. The 5-year survival rates in Finland remain extremely poor, at 6% and 5% for women and men, respectively.

Some variance can be seen in the incidence of pancreatic cancer within Finland (Table 2). According to the Finnish Cancer registry, the highest incidence per 100,000 inhabitants adjusted for age for both sexes between years 2012 - 2016 was in the Helsinki region (22.79). The second highest rates were observed in Turku region (22.56). The lowest rates were observed in the Tampere region (19.56), Kuopio and Oulu being in the middle (20.57 and 21.35, respectively). The highest incidence rate for male was observed in the Helsinki region (26.63), and the lowest in Tampere region (21.21). Highest incidence rate for female was seen in Turku region (21.00) and lowest in Tampere (18.17).
Table 2. Incidence of pancreatic cancer in Finland per 100 000 inhabitants, adjusted for age between years 2012 – 2016, Finnish Cancer Registry.

<table>
<thead>
<tr>
<th>Region</th>
<th>All</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helsinki</td>
<td>22.79</td>
<td>26.63</td>
<td>19.98</td>
</tr>
<tr>
<td>Tampere</td>
<td>19.65</td>
<td>21.21</td>
<td>18.17</td>
</tr>
<tr>
<td>Turku</td>
<td>22.56</td>
<td>24.31</td>
<td>21.00</td>
</tr>
<tr>
<td>Kuopio</td>
<td>20.57</td>
<td>22.54</td>
<td>18.74</td>
</tr>
<tr>
<td>Oulu</td>
<td>21.35</td>
<td>24.75</td>
<td>18.30</td>
</tr>
</tbody>
</table>

2.1.3 Staging

Previously, pancreatic cancer was divided into stages according to the American Joint Committee on Cancer 7th edition guidelines (Edge & Compton, 2010) (Table 3). After that, a newer edition was published in 2017 (Amin et al., 2017) (Table 4). This new edition included changes in the diameter of the tumor and limitation to pancreas. Furthermore, the new division into N1 and N2 was added. The new staging system was validated in a study including 1551 patients (Allen et al., 2017). Below are presented both the 7th and 8th edition of the staging system.

Table 3. Pancreatic cancer TNM staging system (AJCC; 7th edition 2010).

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumor</td>
<td></td>
</tr>
<tr>
<td>Tx</td>
<td>The main tumor cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumor</td>
</tr>
<tr>
<td>Tis</td>
<td>Tumor in situ</td>
</tr>
<tr>
<td>T1</td>
<td>Tumor &lt;2cm across and has not grown outside the pancreas</td>
</tr>
<tr>
<td>T2</td>
<td>Tumor &gt;2cm across but has not grown outside the pancreas</td>
</tr>
<tr>
<td>T3</td>
<td>Tumor has grown outside the pancreas but not into nearby surrounding</td>
</tr>
<tr>
<td></td>
<td>structures or major blood vessels or nerves</td>
</tr>
<tr>
<td>T4</td>
<td>Tumor has grown into nearby large blood vessels or nerves</td>
</tr>
<tr>
<td>Regional lymph nodes</td>
<td></td>
</tr>
<tr>
<td>Nx</td>
<td>Lymph nodes cannot be assessed</td>
</tr>
<tr>
<td>N0</td>
<td>No regional lymph node metastases</td>
</tr>
<tr>
<td>N1</td>
<td>Cancer has spread into nearby lymph nodes</td>
</tr>
<tr>
<td>Distant metastases</td>
<td></td>
</tr>
<tr>
<td>Mx</td>
<td>Distant metastases cannot be assessed</td>
</tr>
<tr>
<td>M0</td>
<td>No distant metastases</td>
</tr>
<tr>
<td>M1</td>
<td>Cancer has spread to distant lymph nodes or distant organs</td>
</tr>
</tbody>
</table>
Table 4. Pancreatic cancer TNM staging system (AJCC; 8th edition 2018).

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumor</td>
<td></td>
</tr>
<tr>
<td>T&lt;sub&gt;x&lt;/sub&gt;</td>
<td>The main tumor cannot be assessed</td>
</tr>
<tr>
<td>T&lt;sub&gt;0&lt;/sub&gt;</td>
<td>No evidence of primary tumor</td>
</tr>
<tr>
<td>T&lt;sub&gt;is&lt;/sub&gt;</td>
<td>Tumor in situ</td>
</tr>
<tr>
<td>T&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Tumor diameter ≤2 cm</td>
</tr>
<tr>
<td>T&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Tumor diameter &gt;2, ≤4 cm</td>
</tr>
<tr>
<td>T&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Tumor diameter &gt;4 cm</td>
</tr>
<tr>
<td>T&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Tumor involvement in celiac axis, common hepatic artery of the superior mesenteric artery</td>
</tr>
</tbody>
</table>

Regional lymph nodes

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>N&lt;sub&gt;x&lt;/sub&gt;</td>
<td>Lymph nodes cannot be assessed</td>
</tr>
<tr>
<td>N&lt;sub&gt;0&lt;/sub&gt;</td>
<td>No regional lymph node metastases</td>
</tr>
<tr>
<td>N&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Cancer has spread into 1-3 regional lymph nodes</td>
</tr>
<tr>
<td>N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Metastasis in over 4 regional lymph nodes</td>
</tr>
</tbody>
</table>

Distant metastases

<table>
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<td>M&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Cancer has spread to distant lymph nodes or distant organs</td>
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Table 5. Stage grouping of pancreatic cancer (AJCC 7th edition 2010).

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2.1.4 Diagnosis

The diagnosis of pancreatic cancer consists of different methods that substantiate each other. These methods include blood tests, various imaging methods and endoscopic interventions.

**Tumor markers in serum:** Currently, the most used blood tests in the diagnosis of pancreatic cancer are carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA) (X. G. Ni et al., 2005). CA19-9 has a sensitivity of 80%, but specificity of only 43%. The combined use of both CA19-9 and CEA increases the specificity substantially, for up to 84% but decreases the sensitivity to 37% as compared to using CA19-9 as the only marker (X. G. Ni et al., 2005).

**Transabdominal ultrasound** is routinely used in the diagnosis of various abdominal emergences. It is highly dependent on the operator’s experience level as well as factor associated with the patient. These factors include abdominal obesity and bowel gas. Considering that there are so many variables, the specificity and sensitivity of the transabdominal ultrasound varies (Conrad & Fernandez-Del Castillo, 2013).

**Computed tomography or CT scan** is routinely applied in the diagnosis of pancreatic cancer. It can be further used in the evaluation of the resectability of the tumor, vascular invasion and diagnosis of any metastatic lesions (Y. Zhang, Huang, Chen, & Jiao, 2012). With modern equipment, CT scan has an overall sensitivity of 96% in detecting pancreatic cancer (Chu, Goggins, & Fishman, 2017).

**Magnetic resonance imaging or MRI** relies on the radio-frequency signal emitted by hydrogen atoms resonating in the magnetic field. According to studies, MRI has around the same sensitivity as CT scan (93.5%) in detecting pancreatic cancer (Chu et al., 2017). Magnetic resonance cholangiopancreatography or MRCP is used to investigate any abnormalities in the pancreatic duct and biliary tract. The method is non-invasive (Maccioni et al., 2010). Enhancing MRI imaging with radiocontrast agent such as gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid, the accuracy of MRI in detecting liver metastases increases to 90.2% (Ito et al., 2017).

**Endoscopic retrograde cholangiopancreatography or ERCP** is applied in the evaluation of pancreatic ducts and biliary tract abnormalities. Compared to MRCP, this method is invasive as it involves the use of an endoscope. This method allows taking cytological samples by brushing or aspiration. According to studies, ERCP improves the diagnostic accuracy in pancreatic cancer (Yamaguchi et al., 2012). Pancreatography allows for visualization and biopsies of intraductal lesions.
of pancreatico-biliary system, and may be useful in determining the optimal surgical intervention (Tyberg et al., 2018).

**Endoscopic ultrasonography** involves the use of an endoscope equipped with an ultrasound probe. This method is also dependent on the experience level of the operator. According to various studies, it might have slightly better accuracy than CT scan in the diagnosis of pancreatic cancer, and in a meta-analysis, a sensitivity of 94% and a specificity of 89% was reported (Gong, Hu, & Zhu, 2012). Endoscopic ultrasound can also be used to take fine-needle aspirates and biopsies for histological examination (Kalogeraki et al., 2016).

### 2.1.5 Treatment

Currently, the only potentially curative treatment option for pancreatic cancer is surgery with oncological treatment; however, only approximately 20% of patients are diagnosed at the early stage, making them eligible for surgical treatment (Khorana et al., 2016; Zhan et al., 2015). A vast majority, around 65% of the patients eligible for surgical treatment, are diagnosed with IIB stage cancer (Hartwig et al., 2011). The most common types of surgical techniques for operating pancreatic cancer include pancreaticoduodenectomy or “Whipple procedure”, distal pancreatectomy and total pancreatectomy. Laparoscopic approach can also be applied, but only in a number of specialized centers (Shin et al., 2015). All of these operation types are demanding both for the patient and the surgeon as they typically include removal of parts of the pancreas, duodenum, proximal jejunum, gallbladder, and in some cases, parts of the stomach.

Since 1997, gemcitabine has been the standard drug of choice for first-line chemotherapy in pancreatic cancer (Burris et al., 1997). The effect of gemcitabine has been since shown (Neoptolemos et al., 2012). In the CONKO-001 study, the gemcitabine administered after the surgery increased the overall survival as well as disease-free survival (Oettle et al., 2013). Since then, new options for oncological treatment have been established. The combination of gemcitabine with capecitabine showed improved survival rates, however, with increased adverse effects (Neoptolemos et al., 2017). Since then there have been multiple attempts to find other options or an effective combination therapy with gemcitabine. One of the most promising was FOLFIRINOX, with improved response, progression-free survival and overall survival; however, this treatment modality had significantly more adverse effects (Conroy et al., 2011). Combination of nanoparticle albumin-bound paclitaxel (nab-paclitaxel) with gemcitabine showed promising results and
had more manageable side effects compared to FOLFIRINOX (Von Hoff et al., 2013). The use of 5-fluorouracil and folinic acid showed improved survival times compared to observation alone (Neoptolemos et al., 2009; Neoptolemos et al., 2012).

The use of radiation therapy in the treatment of locally advanced pancreatic cancer is currently under debate and definitive results of its benefit are sparse (Loehrer PJ et al., 2011; Takaori et al., 2016).

The effect of neoadjuvant therapy in the treatment of pancreatic cancer has been under debate. Advantages of neoadjuvant treatment include down staging of local tumors and increased possibility for complete R0 resection. However, it has also been discussed whether neoadjuvant therapy delays the onset of surgical approaches, and in some cases, makes the surgical treatment impossible due to advanced disease. In the recent years, increasing evidence suggests the use of neoadjuvant therapy as an addition to the regular surgical treatment, as it has been shown to increase disease-free survival as well as disease-specific survival (Jang et al., 2018; Nurmi et al., 2018). The neoadjuvant therapy typically consists of gemcitabine alone or combined with capecitabine, FOLFIRINOX, cisplatin or nab-paclitaxel, combined with radiation treatment.

As a cancer with such a poor prognosis, palliative care is evidently important in pancreatic cancer. Endoscopic, surgical and radiological interventions are applied in the treatment of obstructive jaundice and duodenal obstruction.

### 2.1.6 Prognosis

Even with modern treatment methods and earlier diagnosis, the prognosis of pancreatic cancer remains very poor, however some increase has been seen in the recent years. The general 5-year survival estimates vary from 5-15% depending on the region (Allemani et al., 2018). However, the difference in survival rates between different countries is typically very minimal compared to many other cancers. Survival rates of 10-15% were seen in western countries, Scandinavia, China, Korea and European countries. In Russia, the survival was only around 4%. According to the CONCORD-3 study, some improvement in survival between years 2000 and 2014 was seen in North America and Europe, where survival rates increased around 3-5% (Allemani et al., 2018).

In Finland, the centralization of pancreatic surgery has brought promising increases in survival rates in patients with surgically treated pancreatic cancer. The 5-year survival rate was around 22%, and for R0, local tumors, the 5-year survival
rates were as high as 49% (Seppanen et al., 2017). With the application of neoadjuvant therapy, the results for higher stage pancreatic cancers have shown promising increase in survival rates (Nurmi et al., 2018).

### 2.1.7 Histopathological and surgical prognostic factors

Histopathological and surgical prognostic factors consist of variables that can be visualized by pathological examination or during surgical procedures. Most of the histopathological prognostic factors can be reliably defined from routine surgical specimen using routine pathological diagnostic methods, i.e. microscopic examination (Bilici, 2014).

#### Tumor size

Tumor size is included in the T1 and T2 tumors of the TNM 7th staging system and in T1, T2 and T3 in the 8th staging system of pancreatic cancer. Tumor size can be reliably determined preoperatively, and it has a profound effect on the planning of the surgical intervention. Several studies have shown that larger tumors generally indicate worse prognosis (Akerberg, Ansari, & Andersson, 2016). However, it has been also noticed that a small tumor with a diameter under 1 cm may indicate more rapid progression and therefore have worse prognosis compared to larger tumors (Franko, Hugec, Lopes, & Goldman, 2013; Hur et al., 2016; Izumi, Nakamura, Mano, & Onoda, 2014; Shin et al., 2014). Therefore, there are some limitations to the use of tumor size as a prognostic factor in pancreatic cancer.

#### Lymph nodes

The tumor spread into the nearby lymph nodes is included in the TNM staging system of pancreatic cancer and has been used in the evaluation of prognosis. Both of the staging systems are presented above in Tables 1, 2, 3 and 4. While the sole presence of lymph node metastases can be used as a prognostic factor, lymph node ratio has been shown to describe the prognosis more accurately (Berger, Watson, Ross, & Hoffman, 2004; Riediger et al., 2009; Valsangkar et al., 2013). Lymph node ratio can be determined simply by dividing the number of tumor positive lymph nodes with the number of lymph nodes examined in total.
Surgical margin

The success rate of the surgical intervention depends on the size and localization of the tumor. If the tumor is restricted to the pancreas, it usually indicates better results for surgery. The surgical margin can be divided into three categories according to the tumor growth in the resection marginal. R0 resection means that there is 1 mm of healthy tissue margin detected in the resection marginal, whereas R1 resection means that there is still tumor growth detected with microscopic examination in the resection margin (Esposito et al., 2008). R2 means that there is tumor growth in the resection margin that can be seen with naked eye. Evidence suggests that patients who have undergone R0 resection have significantly better prognosis compared to those who still have tumor growth in the resection marginal (Andren-Sandberg, 2012; Raut et al., 2007).

Perineural and blood vessel invasion

Tumor spreading into the nerves within the pancreatic tissue is one of the causes for persistent pain in pancreatic cancer. Tumor cells can be detected in the neural structures by microscopic examination. Both perineural invasion and blood vessel invasion have been shown to associate with poor overall survival of the patients as well as with lymph node spreading (S. R. Lee, Kim, Son, Yoo, & Shin, 2013; P. H. Wang, Song, Shi, Zhang, & Chen, 2013).

Localization of the tumor

The localization of the tumor can be divided into regions that are the head and neck of the pancreas and the body and tail of the pancreas. Studies have investigated whether the localization of the tumor within the pancreas has effect on the prognosis. One study suggested that patients who had pancreatic tumor in the body and tail of the pancreas had significantly worse survival rates compared to patients who had the tumor in the head and neck of the pancreas. One possible explanation to this is that tumors of the tail can grow larger and longer without showing any symptoms, or the symptoms may be unspecific such as back pain (D. X. Zhang, Dai, Yuan, & Tao, 2012). However, as opposing results also exists, the matter is still under discussion and no consensus has been reached (K. J. Lee et al., 2013; J. K. Park et al., 2008).
**Tumor grade**

The WHO grading system for pancreatic ductal adenocarcinoma is based on a proposition made in 1985 by Kloppel et al. (Kloppel, Lingenthal, von Bulow, & Kern, 1985). Tumor grade measures the differentiation of the tumor. The system considers nuclear atypia, mitotic activity, glandular differentiation and mucin production. Several studies have shown its usefulness as a prognostic factor in pancreatic cancer (Benassai et al., 2000; Gebhardt, Meyer, Reichel, & Wunsch, 2000; Kuhlmann et al., 2004; Lim, Chien, & Earle, 2003), and a larger study by Wasif et al. including over 8,000 patients suggested that tumor grade could be an important prognostic factor in pancreatic ductal adenocarcinoma (Wasif et al., 2010).

### 2.1.8 Molecular and immunohistochemical prognostic factors

Research aimed at finding potential molecular and immunohistological prognostic factors has been going on for decades, and in recent years, some possible markers have been identified and suggested. Some of these markers have even been taken into practice. Harsha et al. recorded over 1,000 potential markers elevated in pancreatic cancer, and there is thus still a lot of research to be done in order to find the most relevant prognostic markers (Harsha et al., 2009). The currently most potential and studied markers are presented below.

**CA19-9**

CA19-9 is currently the most established clinically used blood test in evaluating tumor burden in pancreatic cancer (Jazieh, Foote, & Diaz, 2014). CA19-9 is typically elevated in the serum or plasma of patients with pancreatic or gastrointestinal cancer (Magnani, Steplewski, Koprowski, & Ginsburg, 1983). According to a review by Duffy et al., CA19-9 has a sensitivity of 70–90% and specificity of 90%, making it somewhat useful in the diagnosis of pancreatic tumors and monitoring possible recurrence (M. J. Duffy et al., 2010). However, it cannot be used alone in the primary diagnosis or screening of pancreatic cancer as a substantial proportion of patients do not express CA19-9, which is why other diagnostic methods are needed (Narimatsu et al., 1998). It has been reported that elevated CA19-9 levels seem to have impact on overall patient survival in pancreatic cancer (J. K. Park et al., 2008). However, as approximately 5-10% of
patients are so called Lewis negative, which means they secrete little or no CA19-9, other methods are also needed (Luo et al., 2017). Therefore, it has been suggested that CEA and CA125 should be routinely measured from all patients with pancreatic cancer.

VEGF

Vascular endothelial growth factor has been proposed as one of the most promising immunohistochemical prognostic factors in pancreatic cancer. Positive VEGF expression indicates adverse prognosis in pancreatic cancer. (Smith, Tang, Tudur-Smith, Neoptolemos, & Ghaneh, 2011).

Other immunohistochemical and molecular prognostic factors

Other potential immunohistochemical prognostic factors for pancreatic cancer include p53, p16, SMAD4, bcl-2 and epidermal growth factor receptor (EGFR) (Bold et al., 1999; DiGiuseppe et al., 1994; Naka et al., 1998; Tascilar et al., 2001; Ueda et al., 2004). In single studies, they have been suggested as potential prognostic factors; however, in a meta-analysis by Smith et al. they failed to represent significant prognostic factors in pancreatic cancer (Smith et al., 2011). In the recent years, new promising markers for pancreatic cancer has been suggested. Podocalyxin-like 1, a transmembrane glyco-protein associated with T-class and perineural invasion and was an independent prognostic factor for pancreatic cancer (Saukkonen et al., 2015). Factors involved in the Wnt/β-catenin pathway associated with tumor grade and were independent prognostic factors (Saukkonen et al., 2016). Regenerating islet-derived protein 4 (REG4), a protein typically upregulated in various gastrointestinal diseases, found in the serum and in the pancreatic tissue was also associated with worse survival in early stage pancreatic cancer, and also showed to be useful in the differential diagnosis of pancreatitis from pancreatic cancer (Saukkonen et al., 2018). These new markers show positive results, and further evidence is needed to establish their potential as prognostic markers.

Liquid biopsies

In the recent years, new options for early detection, staging and prognosis have been suggested. One of these possible options is so-called liquid biopsies. Circulating tumor cells, cell-free nucleic acids and noncoding RNA have been
found in the blood of patients with pancreatic cancer (Imamura et al., 2016). Liquid biopsies allow for non-invasive measures for detecting and monitoring pancreatic cancer. Various studies have investigated their usefulness as a tool for early detection and prognosis, and promising results have emerged (Imamura et al., 2016). However, before applying these to the clinical use, some problems must be solved. Methodological and technical aspects vary between studies, and no consensus has been reached. In the future, as more evidence is piling up, liquid biopsies offer one possible addition to the diagnostic and prognostic aspects in pancreatic cancer.

### 2.1.9 Pancreatic intraepithelial neoplasia

Pancreatic cancer arises from microscopic lesions called pancreatic intraepithelial neoplasia (PanIN) (Scarlett, Salisbury, Biankin, & Kench, 2011). PanIN lesions cannot be reliably detected with current radiological imaging methods. PanINs are graded into three grades according to their histological severity (Hruban et al., 2001). PanIN1 lesions represent the lower end spectrum of histological changes whereas PanIN3 lesions contain severe dysplasia.

Typically, PanIN lesions arise from smaller pancreatic ducts (Singh & Maitra, 2007). Due to the practical difficulty of taking biopsies from the pancreas given its retroperitoneal position, it is currently unknown at which point these precursor changes develop. It is suggested that these changes may be present years before the emergence of invasive cancer. It has been estimated that the prevalence of PanIN1 lesions in general population could be relatively high. In a single study, the prevalence of PanIN1 lesions in autopsy material was as high as 77% (Matsuda et al., 2017).
Fig. 1. Hematoxylin-eosin images of different PanIN grades taken from our own material. 
a) PanIN1 b) PanIN2 c) PanIN3.
Histology

Typical morphological changes of pancreatic intraepithelial neoplasia include tall, columnar cells with basally located nuclei and micropapillary architecture. Lesions that are today called PanINs were described well over a 100 years ago; however, until the 21st century, they lacked a proper classification system. In 1999 a histological grading system was established according to the proposition made earlier by Klimstra and Longnecker (Hruban et al., 2004; Klimstra & Longnecker, 1994). PanIN1 lesions contain only a minimal degree of dysplasia. PanIN2 changes contain loss of cell polarity, nuclear crowding, hyperchromatism and enlarged nuclei, representing a moderate degree of dysplasia. Mitotic activity is, however, rarely seen in PanIN2 lesions. The most severe of the lesions, PanIN3, is also sometimes referred to as “carcinoma in situ” since it contains atypical mitoses, luminal necrosis and cribriforming, representing a severe degree of dysplasia. Despite their aggressive histology, PanIN3 lesions do not invade through the basement membrane (Singh & Maitra, 2007). The spectrum of dysplasia and typical characteristics for each PanIN grade are illustrated in Figure 1.

2.1.10 Molecular changes in pancreatic cancer pathogenesis

The pathogenesis of pancreatic cancer involves various alterations in oncogenes and tumor suppressor genes, and many of these changes can be seen already in the early stage PanIN. Below are presented a few of the currently most relevant molecular alterations observed in pancreatic cancer pathogenesis.

KRAS

Approximately 90% of human pancreatic cancers harbor a mutation in the oncogene KRAS (Almoguera et al., 1988; Wood & Hruban, 2012). Mutations in the KRAS are responsible for impractical signaling leading to uncontrolled cancer cell growth, as KRAS encodes the small GTPase, a factor responsible for mediating the signaling cascade downstream of growth factor receptors (Vogelstein & Kinzler, 2004). Typically, the mutations in the KRAS can already be seen in early stage pancreatic cancers and even in the precursor lesions (Caldas & Kern, 1995). The frequency of KRAS mutations increase linearly towards higher grade PanIN (Liu et al., 2016; Lohr, Kloppel, Maisonneuve, Lowenfels, & Luttges, 2005).
CDKN2A

The most frequently mutated tumor suppressor gene in pancreatic cancer is Cyclin-dependent kinase inhibitor 2A (CDKN2A) (Wood & Hruban, 2012). CDKN2A functions in the tumor suppression by affecting the cell cycle. Loss of p16 function, encoded by CDKN2A/INK4A gene, is visible in approximately 90% of pancreatic cancer, and it can be detected with immunostaining in the nucleus (Caldas et al., 1994; Jones et al., 2008). It is also detected in pancreatic intraepithelial neoplasia and approximately 71% of PanIN3 lesions containing loss of nuclear p16 expression (Wilentz et al., 1998; Zinczuk et al., 2018).

SMAD4

The inactivation of tumor suppressor gene SMAD4 is a common occurrence in pancreatic cancer, and it has been estimated that approximately 50% of the pancreatic cancers have this deletion (Cowgill & Muscarella, 2003). This can be detected with immunohistochemical methods (Hahn et al., 1996; Saki & Horii, 2014). Typically, the deletion in SMAD4 occurs in the late stages of tumorigenesis. SMAD4 has a crucial role in the inhibition of cell growth (Massague, 1998). Loss of SMAD4 interferes with the transforming growth factor β (TGF-β) family of cell surface receptors and causes uncontrolled proliferation and decreased growth inhibition. Loss of SMAD4 expression can be detected in around 41-46% of PanIN3 lesions (Hutchings et al., 2018; Wilentz et al., 2000).

P53

The TP53 gene codes for the protein p53. Tumor suppressor gene p53 is involved in the regulation of cell cycle, and furthermore, in the induction of apoptosis (Surget, Khoury, & Bourdon, 2013). Once inactivated, p53 allows cancer cells to grow rapidly and survive significantly longer. Alterations in the p53 protein allow cells to avoid apoptotic signals and DNA damage checkpoints, and the loss of p53 has been suggested to play an important role in genomic instability in pancreatic cancer (Hingorani et al., 2005). Approximately 50% of pancreatic cancers have mutation in the p53 gene (Li, Bhuiyan, Vaitkevicius, & Sarkar, 1998). P53 can be immunohistochemically detected in the nuclei of PanIN3 (Maitra et al., 2003; Zinczuk et al., 2018).
2.2 Innate immunity

The human immune system can be divided into innate immunity system and adaptive immunity system. The innate immunity system acts against invading pathogens using generic reactions, and unlike adaptive immunity, innate immunity does not provide long-lasting immunity against certain pathogens. The innate immunity system functions in the frontline of immunity, reacting to the invading pathogens much more quickly than adaptive immunity. Compared to adaptive immunity, innate immunity is a system that is much more primitive. Innate immunity consists of leukocytes such as neutrophils, basophils, eosinophils, macrophages, dendritic cells, mast cells and natural killer cells, as well as the complement system and pattern-recognizing receptors. Normal healthy skin, acidity of the stomach and mucous membrane can also be recognized as part of the innate immunity system (Hoffmann & Akira, 2013). Cells of the innate immunity system recognize pathogens from host structures through pattern-recognizing receptors (PRRs). PRRs recognize structures of the pathogens, such as cellular membrane components and certain DNA strands (Akira, Uematsu, & Takeuchi, 2006). These structures are referred to as pathogen-associated molecular patterns or PAMPs.

2.2.1 Pattern-recognizing receptors

Pattern-recognizing receptors are expressed in the cells of the innate immunity system, such as neutrophils, macrophages and dendritic cells, as well as in the epithelial cells of various organs and structures of the mammal body. PRRs recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) which derive from host cells after cell damage or death. Activation of PRRs leads to release of inflammatory cytokines and antigen-specific adaptive immune response (Kumar, Kawai, & Akira, 2011). Different PRRs react to different PAMPs and activate signaling pathways that lead to distinct antipathogen responses. PAMPs recognized by PRRs include bacterial or viral DNA or RNA, bacterial peptides such as flagellin, peptidoglycans and lipoteichoic acids from Gram-positive bacteria and bacterial carbohydrates such as lipopolysaccharides (LPS) (Akira et al., 2006; Belvin & Anderson, 1996; Garg et al., 2015; Kumar et al., 2011). According to their localization in the cell, PRRs can be divided to cytoplasmic bound PRRs that include NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) (Franchi, Warner, Viani, & Nunez, 2009;
Takeuchi & Akira, 2010), and to membrane bound PRRs including C-type lectin receptors (CLRs) and Toll-like receptors (TLRs) (Beutler et al., 2006; Geijtenbeek & Gringhuis, 2009; Takeda, Kaisho, & Akira, 2003).

2.2.2 Toll-like receptors

Toll-like receptors (TLRs) are pattern-recognizing receptors of the innate immunity. They were originally described in Drosophila in 1985 (Anderson, Bokla, & Nusslein-Volhard, 1985). To date, 13 TLRs have been identified in mammals, and ten of them are found in humans (Akira et al., 2006; K. Chen et al., 2007; Takeda et al., 2003). At first TLRs were found in the membrane of the innate immunity system cells such as macrophages and monocytes, but as research progressed, they were discovered in the epithelial cells of various organs. In recent years, they have been detected in various malignancies and cancers. Their involvement in cancer progression and function as prognostic factors in many different cancer types is becoming evident.

2.2.3 TLR signaling

Activation of TLRs by PAMPs leads to initiation of a signaling cascade that ultimately leads to response against pathogens. There are two main pathways through which the TLR signaling cascade advances, and these pathways are divided according to the myeloid differentiation primary-response protein 88 (MyD88) to the MyD88-dependent pathway and MyD88-independent pathway (also known as TRIF-dependent pathway). Both of these pathways ultimately lead to activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK), which initiate the immune responses towards the invading pathogens.

In the next two chapters are presented short overviews of both of the pathways through which TLRs biological functions are initiated. The presented signaling cascades are still incomplete and it has been presented that alternative signaling cascades for TLRs that need further investigation also exist. The signaling cascade is illustrated in the Figure 2.
**MyD88 dependent pathway**

All of the TLRs except TLR3 use the MyD88 pathway. MyD88 functions as an adaptor molecule for the TLR signaling pathway. Upon activation with a specific ligand, TLR interacts with MyD88, which triggers a signaling cascade that ultimately initiates functions specific for each TLR. When activated, MyD88 recruits interleukin-1 receptor-associated kinases (IRAKs) IRAK1, IRAK2 and IRAK4. IRAK activation leads to phosphorylation and activation of TNF receptor associated factor 6 (TRAF6). TRAF6 then polyubiquinates the transforming growth factor beta-activated kinase 1 (TAK1). This polyubiquination is activated through Lys63 (K63), which binds to the regulatory parts of the TAK1; these are called TAB2 and TAB3. Upon activation, TAK1 activates MAPK, a kinase responsible for the production of various inflammatory cytokines. The K63 also binds to the binding domain of NEMO, which then modulates the IkB kinase (IKK), a complex involved in NF-κB activation. Activation of NF-κB allows it to diffuse into the nucleus where it initiates transcription of inflammatory cytokines such as IL-6, IL-12 and tumor necrosis factor alpha (TNF-α) (Bhoj & Chen, 2009; Kawagoe et al., 2008; Kawai & Akira, 2010; Rahighi et al., 2009; Yamamoto et al., 2006).

**MyD88-independent pathway**

TLR3 uses the MyD88-independent pathway, and TLR4 uses both the MyD88-dependent and -independent pathways. Specific ligand activates the TLR, which leads to recruitment of the TIR-domain-containing adapter-inducing interferon-β (TRIF). TRIF functions as an adaptor. TRIF activates both Receptor-interacting protein 1 (RIP1) and TANK-binding kinase 1 (TBK1), which create alternative signaling pathways. Activation of RIP1 leads to polyubiquitination and activation of TAK1, and further to activation of NF-κB, similarly to the MyD88-dependent pathway. The alternative signaling cascade goes through the activation of Interferon regulatory factor 3 (IRF3) by the signaling complex TRIF/TBK1. IRF3 is phosphorylated, allowing it to transfer to the nucleus where it initiates the transcription of interferon-β (Ermolaeva et al., 2008; Kawagoe et al., 2008; Kawai & Akira, 2010; Oganesyan et al., 2006).
Fig. 2. Schematic overview of TLR signaling cascades. Picture modified from Kawai and Akira 2010.

2.2.4 Toll-like receptor 2

Toll-like receptor 2 (TLR2) is coded by the TLR2 gene located in human chromosome 4. TLR2 is expressed in various cells of the immunity system, such as macrophages, dendritic cells, B-cells and T-cells. In addition to that, TLR2 can be found in the epithelial cells of airways (Cario, 2008). TLR2 is located on the cell surface. Given its membranous localization, TLR2 recognizes lipoproteins from Gram-negative bacteria, peptidoglycans and lipoteichoic acid from Gram-positive bacteria, and zymosan from fungi. Furthermore, it recognizes endogenous ligands released from host cells by mechanical or chemical trauma (Aliprantis et al., 1999; Aliprantis, Yang, Weiss, Godowski, & Zychlinsky, 2000; Brightbill et al., 1999; Cheng, He, Tian, Ye, & Ye, 2008; Lien et al., 1999; Schwandner, Dziarski, Wesche, Rothe, & Kirschning, 1999; Underhill, Ozinsky, Smith, & Aderem, 1999; Yoshimura et al., 1999). TLR2 forms dimers with TLR1 and TLR6 (Ozinsky et al., 2000). Increased TLR2 expression is found in various malignancies of the gastrointestinal tract, such as esophageal cancer and Barrett’s esophagus, gastric...
cancer and colorectal cancer (Huhta, Helminen, Lehenkari et al., 2016; Nihon-Yanagi, Terai, Murano, Matsumoto, & Okazumi, 2012; H. Yang et al., 2014).

2.2.5 Toll-like receptor 4

Toll-like receptor 4 (TLR4) is coded by the TLR4 gene located in chromosome 9 in humans. TLR4 is located on the cell surface and in the endosomes. TLR4 was first discovered as a lipopolysaccharide (LPS)-sensing receptor (Hoshino et al., 1999). LPS is a component of the cell wall in Gram-negative bacteria. Since then there have been many discoveries of other ligands of TLR4 as well. To date, TLR4 is also known to recognize fusion proteins derived from RS virus and heat shock proteins released from the host by ultraviolet radiation and viral and bacterial infections (Haynes et al., 2001; Kurt-Jones et al., 2000; Ohashi, Burkart, Flohe, & Kolb, 2000). Increasing evidence suggests that TLR4 plays an important part in various malignancies and cancers of the gastrointestinal tract. Abnormal levels of TLR4 have been found in hepatocellular carcinoma, gastric cancer, colorectal cancer, Barrett’s esophagus and esophageal cancer (Eiro, Gonzalez et al., 2013; Fernandez-Garcia, Eiro, Gonzalez-Reyes et al., 2014; Huhta et al., 2016; Verbeek et al., 2014).

2.2.6 Toll-like receptor 9

Toll-like receptor 9 (TLR9) is coded by the TLR9 gene located in human chromosome 3. TLR9 is located in the endoplasmic reticulum of the cell in its inactive state. At first, TLR9 expression was found in the innate immunity system cells such as macrophages, natural killer cells and dendritic cells, but since then it has also been found in epithelial cells, stem cells, fibroblasts, muscle cells and glial cells. TLR9 is activated in the endolysosomal compartment by cleavage of the C- and N-termini (B. Park et al., 2008). TLR9 recognizes unmethylated CpG DNA of the bacteria as well as methylated CpG DNA (Hemmi et al., 2000). Increased TLR9 expression has been found in malignancies of the gastrointestinal tract. For example, hepatocellular cancer, colon cancer, esophageal cancer and gastric cancer all show elevated levels of TLR9, and it also seems to have prognostic effect in some of these cancer types (Fernandez-Garcia et al., 2014; Furi et al., 2013; Kauppila et al., 2011; Nojiri et al., 2013; Tanaka et al., 2010).
2.2.7 TLRs and cancer promotion and regression

Infections and various inflammatory states have been linked to cancer development. The first observations were made as early as in 1881 by Virchow who observed leukocytes in neoplastic tissues (Virchow, 1881). The most well-known examples may be links between H.Pylorii and gastric cancer, hepatitis and hepatocellular cancer, and inflammatory bowel disease and colon cancer (Graham, 2014; Waldner & Neurath, 2009).

The involvement of Toll-like receptors in various cancers is becoming increasingly evident. In addition to recognizing PAMPs, TLRs also recognize a large variety of DAMPs, making them regulators in a wide range of inflammatory and immune responses. According to current knowledge, TLRs function in both the regression and promotion of the tumor (Pradere, Dapito, & Schwabe, 2014). TLRs initiate signaling cascades that ultimately lead to elimination of the pathogens; however, TLR activation can also promote tumorigenesis through proinflammatory, anti-apoptotic and proliferative signaling.

TLR activation leads to induction of transcription factor NF-κB, responsible for regulating the transcription of over 100 proinflammatory genes (Pahl, 1999). NF-κB promotes the induction of cancer-promoting inflammatory cytokines, such as IL-1β, TNFα and IL-6, all of which are known to promote cancers such as intestinal, liver and stomach cancer (Apte & Voronov, 2008; Grivennikov et al., 2009; Tu et al., 2008). Furthermore, the activation of NF-κB stimulates anti-apoptotic effects in the tumor cells (Dutta, Fan, Gupta, Fan, & Gelinás, 2006). NF-κB activation through TLR signaling has been shown to promote anti-apoptotic properties in lung cancer, liver cancer, colon cancer and stomach cancer (Cherfils-Vicini et al., 2010; Dapito et al., 2012; Fukata et al., 2006; Tye et al., 2012).

Dendritic cells function at the border of innate and adaptive immunity. They contain a high number of PRRs such as Toll-like receptors, and once presented with an antigen, they activate local inflammatory responses by secreting cytokines and migrate to lymph nodes where they recruit T cells (Palucka & Banchereau, 2012). Dendritic cells activated by TLRs mediate anti-tumor responses through T cell activation, antigen presentation and direct cytotoxic effects on tumor cells (Garaude, Kent, van Rooijen, & Blander, 2012). However, this activation can be inhibited by inhibitory signals produced by the cancer cells (Palucka & Banchereau, 2012).
2.2.8 TLRs in cancer prognosis

In recent years, the presence of Toll-like receptors in various cancers has become increasingly evident. In the gastrointestinal tract, TLRs participate in the regulation of intestinal inflammation, homeostasis and epithelial cell proliferation (Fukata & Abreu, 2008). To this day, various TLRs have been shown to have prognostic role in various cancer types (Q. Wang et al., 2018).

In organs that are exposed to abundant bacterial loads, such as tongue, esophagus, and colon, high expression of TLRs is typically associated with poor outcome of the patients (Eiro et al., 2013; Huhta et al., 2016; Kauppila et al., 2011; Kauppila et al., 2015; Makinen et al., 2015; E. L. Wang et al., 2010). In these organs, inappropriate responses to local pathogens leads to accumulation of cytokines and inflammatory cells, which ultimately leads to carcinogenesis (Testro & Visvanathan, 2009). Conversely, in organs that are less exposed to bacteria, high TLR9 expression has been linked to better prognosis. Tuomela et al. found that low TLR9 expression was associated with more aggressive subtype of triple-negative breast cancer (Tuomela et al., 2012). In renal cell carcinoma, low TLR9 expression indicated poor prognosis, but no association with clinicopathological markers was found (Ronkainen et al., 2011). High expression of TLR9 associated with good prognosis in carcinoma of salivary glands, and showed a trend for association with better differentiation and lower stage disease (Korvala et al., 2014). The effect of TLRs in different malignancies are summarized in Table 7.

Table 7. The effect of TLRs on patient prognosis in different cancer types.

<table>
<thead>
<tr>
<th>TLR</th>
<th>Cancer type</th>
<th>High expression correlates with</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2</td>
<td>Pancreatic cancer</td>
<td>Good prognosis in stage I-II</td>
<td>Lanki et al., 2018</td>
</tr>
<tr>
<td></td>
<td>Hepatocellular carcinoma</td>
<td>Poor prognosis</td>
<td>Zhe et al., 2016</td>
</tr>
<tr>
<td>TLR4</td>
<td>Colorectal cancer</td>
<td>Poor prognosis</td>
<td>E. L. Wang et al., 2010</td>
</tr>
<tr>
<td></td>
<td>Hepatocellular carcinoma</td>
<td>Poor prognosis</td>
<td>Huhta et al., 2016</td>
</tr>
<tr>
<td></td>
<td>Esophageal adenocarcinoma</td>
<td>Poor prognosis</td>
<td>Eiro, Ovies et al., 2013</td>
</tr>
<tr>
<td></td>
<td>Melanoma</td>
<td>Poor prognosis</td>
<td>J. J. Zhang et al., 2010</td>
</tr>
<tr>
<td></td>
<td>Pancreatic cancer</td>
<td>Good prognosis in stage I-II</td>
<td>Lanki et al., 2018</td>
</tr>
<tr>
<td></td>
<td>Ovarian cancer</td>
<td>Poor prognosis</td>
<td>Kim et al., 2012</td>
</tr>
<tr>
<td></td>
<td>Hepatocellular carcinoma</td>
<td>Poor prognosis with TLR9 staining</td>
<td>Eiro et al., 2014</td>
</tr>
<tr>
<td></td>
<td>Breast cancer</td>
<td>Poor prognosis</td>
<td>F. J. Ma et al., 2014</td>
</tr>
<tr>
<td>TLR5</td>
<td>HPV-positive oropharyngeal squamous cell carcinoma</td>
<td>Poor prognosis</td>
<td>Jouhi et al., 2017</td>
</tr>
<tr>
<td>TLR7</td>
<td>Colorectal cancer</td>
<td>Poor prognosis</td>
<td>Grimm et al., 2010</td>
</tr>
<tr>
<td>TLR</td>
<td>Cancer type</td>
<td>High expression correlates with</td>
<td>Reference</td>
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<tr>
<td></td>
<td>Non-small cell lung cancer</td>
<td>Poor prognosis</td>
<td>Chatterjee et al., 2014</td>
</tr>
<tr>
<td></td>
<td>Oral squamous cell carcinoma</td>
<td>Poor prognosis</td>
<td>Y. H. Ni et al., 2015</td>
</tr>
<tr>
<td></td>
<td>HPV-positive oropharyngeal squamous cell carcinoma</td>
<td>Good prognosis</td>
<td>Jouhi et al., 2017</td>
</tr>
<tr>
<td>TLR8</td>
<td>Colorectal cancer</td>
<td>Poor prognosis</td>
<td>Grimm et al., 2010</td>
</tr>
<tr>
<td></td>
<td>Esophageal adenocarcinoma</td>
<td>Poor prognosis</td>
<td>Helminen et al., 2016</td>
</tr>
<tr>
<td>TLR9</td>
<td>Renal cell carcinoma</td>
<td>Good prognosis</td>
<td>Ronkainen et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Salivary gland carcinoma</td>
<td>Good prognosis</td>
<td>Korvala et al., 2014</td>
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<tr>
<td></td>
<td>Breast cancer</td>
<td>Good prognosis</td>
<td>Tuomela et al., 2012</td>
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<tr>
<td></td>
<td>Squamous cell carcinoma of tongue</td>
<td>Poor prognosis</td>
<td>Kauppila et al., 2015</td>
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<tr>
<td></td>
<td>Esophageal adenocarcinoma</td>
<td>Poor prognosis</td>
<td>Kauppila et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Prostate cancer</td>
<td>Poor prognosis</td>
<td>Vaisanen, Jukkola-Vuorinen, Vuopala, Selander, &amp; Vaarala, 2013</td>
</tr>
<tr>
<td></td>
<td>Chronic lymphocytic leukemia</td>
<td>Good prognosis</td>
<td>Wlasiuk, Tomczak, Zajac, Dmoszynska, &amp; Giannopoulos, 2013</td>
</tr>
</tbody>
</table>

### 2.2.9 TLRs in pancreatic cancer

Current research has shown that Toll-like receptors are widely expressed in pancreatic cancer, and some of them have been suggested as possible therapeutic targets (Vaz & Andersson, 2014). TLR2 is widely expressed in both pancreatic cancer cell lines and in resected pancreatic tumors, being almost absent in normal healthy exocrine pancreatic tissue (Grimmig et al., 2016; Morse et al., 2010). Activation of TLR2 with synthetic lipoprotein Macrophage-activating lipoprotein 2 (MALP-2) has shown promising results in tumor growth reduction and prolonged survival in both murine models and patient trials (Schmidt et al., 2007; Schneider et al., 2004). However, more recent studies showed that TLR2 activation can also have tumor-promoting effects through activation of growth factors such as Vascular endothelial growth factor (VEGF) and Platelet-derived growth factor (PDGF) (Grimmig et al., 2016).

TLR4 is also overexpressed in murine models and human pancreatic cancer (Ikebe et al., 2009; Ochi, Nguyen et al., 2012). However, TLR4 seems to have controversial effects on tumor growth and inhibition depending on which pathway is activated. Inhibition of the MyD88 pathway had tumor-accelerating effects and even lead to a rise of aggressive TP53 mutated pancreatic cancer cells, whereas the
inhibition of TRIF or MyD88-independent pathway had anti-tumor effects. Co-expression of TLR4 with Hypoxia inducible factor-1 alpha (HIF-1alpha) was found in pancreatic cancer and it was suggested that HIF-1alpha overexpression could be responsible for TLR4 expression under hypoxic circumstances (Fan et al., 2012; J. J. Zhang et al., 2010). With the co-expression of HIF-1alpha and TLR4, a decreased survival rate was observed. TLR4 activation also led to an increase in angiogenesis (Sun et al., 2016). In early stage pancreatic cancer, TLR2 and TLR4 associated with improved prognosis (Lanki et al., 2018).

TLR9 appears to be a promising marker for pancreatic cancer. The expression of TLR9 is somewhat higher in pancreatic cancer tissue compared to normal adjacent exocrine pancreas (Wu et al., 2011). Activation of TLR9 with oligodeoxynucleotides that contain CpG motifs (CpG-ODNs) led to anti-tumor immune responses (Wu et al., 2011). CpG-ODNs function as agonists for TLR9. The hypothesis is that CpG-ODNs activate cytotoxic T-cells, NK-cells and dendritic cells, which ultimately leads to initiation of anti-tumoral immune responses (Jacobs et al., 2011). The CpG-ODNs alone had little effect on cancer progression; however, when combined with gemcitabine, the survival times were significantly improved (Pratesi et al., 2005). Zambirinis et al. found contradictory results on TLR9 ligation and tumor promotion (Zambirinis et al., 2015). According to their findings, TLR9 ligation could be oncogenic in pancreatic cancer. They hypothesized that rather than the activation of TLR9 in tumor cells, the activation in pancreatic stellate cells could be more relevant to the tumorigenesis in pancreatic cancer. Activation of TLR9 in pancreatic stellate cells leads to the production of chemokines CCL3 and CCL11 which promote tumor progression in pancreatic cancer. The evidence of TLR9 impact on patient survival is currently limited and additional research is needed to confirm its role as a prognostic factor.

TLR3 and TLR7 have also been investigated in pancreatic cancer. TLR3 was linked to chronic pancreatitis, and in hPDA cell lines TLR3 correlated with tumor cell growth, but no other evidence can be found (Schwartz et al., 2009; Soga et al., 2009). TLR7 is found in the murine and human pancreatic cancer, and appears to regulate carcinogenesis through various signaling mechanisms (Ochi, Graffeo et al., 2012). TLR7 also increased cancer cell proliferation and chemoresistance in vitro, and TLR7 expression increased in advanced stage tumors (Grimmig et al., 2015).
2.2.10 TLRs in pancreatitis

Since TLRs recognize DAMPs in addition to PAMPs, their involvement in acute pancreatitis has been under rigorous research in recent years. Studies have suggested the involvement of TLR2, TLR4 and TLR9 in acute pancreatitis, but research investigating their presence in cancer-related pancreatitis is currently limited. Many of the studies confirm TLRs’ presence in acute pancreatitis in rodents, but in humans, the studies rely mostly on DNA analysis or blood samples. Elevated TLR2mRNA and TLR4mRNA levels were found in rats with cerulein-induced acute pancreatitis and various polymorphisms in TLR2 and TLR4 genes were associated with increased risk for acute pancreatitis (Ding et al., 2013; Gao, Zhou, Li, & Chen, 2007; Takagi et al., 2009). However, in TLR4 the matter appears to be a lot more complicated as studies that confirm no association with TLR4 gene polymorphisms also exist (Guenther et al., 2010; D. Zhang, Zheng, Zhou, Yu, & Li, 2008; Zhou, Cui, Cai, Xiang, & Zhang, 2014). TLR9 was found to be expressed in pancreatic tissue in mice, but this matter is currently in need of further research (Hoque et al., 2011; Zeng et al., 2008).

The understanding of the role of TLRs in chronic pancreatitis is currently very limited. TLR2, TLR4 and TLR9 expression levels were shown to be increased in chronic pancreatitis tissue in very small study sample (n=4) (Grimmig et al., 2016). Therefore further evidence is needed to establish their role in chronic pancreatitis.

2.3 Hypoxia

The human organism relies heavily on oxygen to execute physiological processes. Lack of oxygen leads to hypoxia in a healthy organism. This occurs when the demand of oxygen exceeds the supply. This happens occasionally in healthy organisms, but in addition to normal physiological functions, the involvement of hypoxia has become evident in cancer growth, tumor microenvironment and tumorigenesis (Noman et al., 2015). The rapid growth of the tumor leads to poor nutrient supply and oxygenation in the tumor center (Pastorek & Pastorekova, 2015). Rapidly growing tumor cells demand large quantities of oxygen, and this, together with increased diffusing distances, leads to neovascularization within the tumor (J. P. Duffy, Eibl, Reber, & Hines, 2003). In addition, hypoxia contributes significantly to the formation of aggressive cell types by affecting cell metabolism, cell cycle, gene transcription and invasiveness (Ratcliffé, O'Rourke, Maxwell, & Pugh, 1998).
2.3.1 Hypoxia-inducible factor 1 alpha

Hypoxia-inducible factor 1 alpha (HIF-1alpha) is a protein expressed under hypoxic conditions. It has an important function in maintaining sufficient oxygen levels in normal healthy tissues. HIF-1alpha is encoded by the HIF1A gene, and it forms a heterodimer with the HIF-1beta subunit (Hogenesch et al., 1997). In normoxic conditions, HIF-1alpha is degraded, but under hypoxic conditions, the degradation is inhibited and HIF-1alpha begins to accumulate (Huang, Arany, Livingston, & Bunn, 1996). Activation of HIF-1alpha under hypoxic conditions initiates transcription of various genes, which leads to increased oxygen delivery to the hypoxic region. Perhaps the most well-known of these genes are vascular endothelial growth factor (VEGF) and erythropoietin (J. W. Lee, Bae, Jeong, Kim, & Kim, 2004). It has been well established that HIF-1alpha is overexpressed in various cancer types, and in many cancer types, overexpression has been linked to adverse prognosis (Semenza, 2003; Zhong et al., 1999).

2.3.2 Carbonic anhydrase 9

Carbonic anhydrases are enzymes that function as catalysts for the reaction where carbon dioxide and water are converted to bicarbonate and protons. Carbonic anhydrase 9 (CAIX) is one of these enzymes, and it has been linked to hypoxia (Opavsky et al., 1996). CAIX is located in the membrane of the cells, and it is regulated by HIF-1alpha under hypoxic conditions and typically found in the hypoxic and perinecrotic areas in tumors (Wykoff et al., 2000). Overexpression of CAIX has been linked to poor prognosis in various cancer types (Potter & Harris, 2003).

2.3.3 Hypoxia and pancreatic cancer

Pancreatic cancer is characterized by abundant desmoplastic stroma, hypoxia and neovascularization (Akakura et al., 2001). Dense desmoplastic stroma and the rapid growth of the tumor lead to depletion of oxygen due to increased distances from the nearby blood vessels. Tumor cells that are further away from nearby blood vessels react by increasing the formation of new blood vessels. Hypoxia in pancreatic cancer and other solid tumors functions both by slowing tumor growth and causing apoptosis to maintain homeostasis and by activating responses that lead to elevated resistance to hypoxia and chemoradiation treatments, ultimately
making these cells more malignant (Vaupel, Thews, & Hoeckel, 2001). HIF-1alpha and CAIX expression is found abundantly in pancreatic cancer. In recent years, it has been shown that high HIF-1alpha and CAIX expression is linked to adverse prognosis in pancreatic cancer (Kivela et al., 2000; Li, Dong, Sheng, & Huang, 2016; Ye et al., 2014). A large meta-analysis consisting of eight original publications concluded that high HIF-1alpha associated with adverse prognosis in pancreatic cancer (Ye et al., 2014). This meta-analysis included studies conducted in the Asian countries; currently, results from Western countries are lacking.

2.4 TLR and hypoxia interplay

Persistent inflammatory response has been shown to participate in tumorigenesis in various solid tumors (Mantovani, Allavena, Sica, & Balkwill, 2008). For over a decade, it has been known that hypoxia and persistent inflammatory response interact in the progression of cancer; however, the complete mechanism through which this is achieved remains unclear to date (Han et al., 2016). Many hypotheses have been suggested, one of which in particular seems the most promising. Nuclear factor-κB (NF-κB) is proposed to be the link between hypoxia and inflammatory response (Karin, 2008). At the physiological state NF-κB can be found in almost all cell types and it participates in cellular responses to immune reactions, cytokines and stress. Toll-like receptors mediate the NF-κB signaling among other immune receptors. It was recently shown that hypoxia activates NF-κB signaling in solid tumor microenvironment (Bandarra, Biddlestone, Mudie, Muller, & Rocha, 2015; Gorlach & Bonello, 2008). Therefore, it is believed that NF-κB forms the link between hypoxia and TLR signaling in solid tumors.

Based on these findings, further research has since been conducted. In recent studies, it was shown that there is a feedback loop between HIF, and that HIF-1alpha activates TLR2, TLR6 expression (Han et al., 2016; Kuhlicke, Frick, Morote-Garcia, Rosenberger, & Eltzschig, 2007). In 2010, Zhang et al. discovered a correlation between HIF-1alpha and TLR4 expression in pancreatic cancer, and later, another group found further evidence that HIF-1alpha activates TLR4 expression in hypoxic conditions in pancreatic cancer (Fan et al., 2012; J. J. Zhang et al., 2010). It was also discovered that elevated HIF-1alpha levels in gliomas occurred with decreased TLR9 expression and that activation of TLR9 decreased HIF-1alpha activity through insulin-like growth factor (IGF-1). Conversely, knockdown of HIF-1alpha resulted in decreased TLR9 levels. These findings led the authors to hypothesize that there is a complex feedback-loop system between
HIF-1alpha and TLR9 (Sinha, Koul, Dixit, Sharma, & Sen, 2011). These are all preliminary results, and more research is needed to confirm the interplay between HIF-1alpha and TLR in cancer. However, this might be one potential link between hypoxia and innate immunity activation in tumorigenesis.

2.5 Tumor microenvironment

The tumor microenvironment (TME) consists of fibroblasts, immune and inflammatory cells, lymphocytes, blood vessels and the extracellular matrix (ECM) (Spill, Reynolds, Kamm, & Zaman, 2016). Tumor and TME are in constant interplay as the tumor releases signals that interact with TME. These signals can promote the formation of blood vessels and influence the immune responses in the TME. Vice versa, the TME promotes tumor cell growth and evolution (Bonnans, Chou, & Werb, 2014; Cheresh & Stupp, 2008; de Visser, Eichten, & Coussens, 2006).

ECM consists of proteins such as elastin, collagens, fibronectin and tenascin-C. Instead of being an inanimate structure, ECM is constantly changing as the tumor grows and progresses (Lu, Weaver, & Werb, 2012). Various alterations are seen at different stages of tumor growth from early stages to invasion and metastasis. ECM affects tumor progression by promoting metastasis and transformation of tumor cells through the ECM, and furthermore, the ECM affects the whole TME by inducing inflammatory responses and angiogenesis (Hynes, 2009). In recent years, the role of tumor microenvironment in the pathology and development of various cancers has become increasingly evident, and more and more research is focused on this area of cancer research.

2.5.1 Extracellular matrix in pancreatic cancer

Various solid tumors consist of some kind of stroma, more extensive in some and less in others. Pancreatic cancer can be seen as a cancer at the “more extensive” end of the spectrum, as it is typically characterized by dense stroma. Proteins of the ECM in pancreatic cancer provide structure to the tissue, and in addition to that, it functions in signaling cascades that promote tumor cell migration, proliferation and tumor cell survival (Topalovski & Brekken, 2016). ECM in pancreatic cancer consists of collagens and various proteins such as tenascin-C and fibronectin. The source for most of the proteins in pancreatic cancer ECM are pancreatic stellate cells (PSC). Pancreatic stellate cells are a type of fibroblasts that are activated upon
tissue injury or inflammation (Lunardi, Muschel, & Brunner, 2014). In normal healthy pancreatic tissue, PSCs are in non-functional state. When activated, PSCs begin to express α-smooth muscle actin (SMA), which has been used as a marker for activation of PSCs (Erkan et al., 2008). Previous studies have concluded that activation of PSC and increased expression of SMA is associated with worse patient outcome (Sinn et al., 2014). This has led to the conclusion that ECM plays a vital part in cancer progression in pancreatic ductal adenocarcinoma.

2.5.2 Tenascin-C

Tenascin-C is a glycoprotein of the ECM. Tenascin-C interacts with proteins of the ECM, especially with fibronectin (Chung, Zardi, & Erickson, 1995). Tenascin-C can be found in various developing tissues, such as developing tendons, bone and cartilage. Tenascin-C is involved in many physiological functions of the organism, such as wound healing and reparation of tissue damage, cell proliferation and migration, and embryonal development (Erickson, 1993).

Abundant expression of tenascin-C has been found in the stroma of various solid tumors (Orend & Chiquet-Ehrismann, 2006). Association with poor prognosis can be seen in bladder cancer, clear cell renal carcinoma, colorectal cancer, esophageal squamous cell carcinoma and gastric cancer (Brunner et al., 2004; Lundin, Nordling, Lundin, & Haglund, 2007; Ohno et al., 2008; Wiksten et al., 2003; Z. T. Yang et al., 2016). In pancreatic cancer, tenascin-C correlated with poor differentiation of the tumor, and co-expression with Matrix metalloproteinase-9 (MMP-9) was associated with TNM-stage, metastasis, lymph node invasion, vascular invasion and worsened survival of the patients (Juuti, Nordling, Louhimo, Lundin, & Haglund, 2004; Xu et al., 2015).

In most recent years, it has been discovered that tenascin-C also plays a part in inflammatory response. The matter is currently under discussion, but so far three main components have been identified to interact with tenascin-C, causing the inflammatory effects: two different integrins and Toll-like receptor 4 (Marzeda & Midwood, 2018). The complete mechanism through which tenascin-C activates TLR4 and initiates the inflammatory response is currently unclear.

2.5.3 Fibronectin

Fibronectin is another glycoprotein of the ECM. Fibronectin binds to receptor-proteins of the cell surface called integrins (Pankov & Yamada, 2002). Fibronectin
is mainly produced by fibroblasts. To date, two forms of fibronectin have been identified, the soluble and the insoluble form. The soluble form of fibronectin exists in blood plasma. The insoluble form is a crucial part of normal ECM. In normal healthy tissue, fibronectin is vital in wound healing and development. Various pathologies, such as fibrosis and various cancer types, express altered levels of fibronectin (Williams, Engler, Slone, Galante, & Schwarzbauer, 2008). Previous reports have suggested that stromal overexpression of fibronectin associates with poor prognosis of patients in breast cancer, nasopharyngeal carcinoma, tongue cancer, esophageal squamous cell carcinoma and colorectal cancer (Bae et al., 2013; Fernandez-Garcia, Eiro, Marin et al., 2014; L. J. Ma et al., 2014; Sudo et al., 2013; Sundquist et al., 2017; Yi, Xiao, Ding, Luo, & Yang, 2016).

In pancreatic cancer, fibronectin is widely expressed (Ramaswamy, Ross, Lander, & Golub, 2003). The expression pattern is almost completely limited to the cancerous stroma, and normal healthy pancreatic tissue is typically negative for fibronectin expression (Topalovski & Brekken, 2016). As fibronectin is an important component of the surrounding stroma and abundantly found in the stroma of pancreatic cancer, it has various functions that enhance the overall aggressiveness of the cancer.

In pancreatic cancer, fibronectin promotes cell migration, invasion and metastasis (Akiyama, Olden, & Yamada, 1995). This is achieved through different mechanisms, one of which is the induction of epithelial-mesenchymal transition (EMT). EMT is a characteristic of cancer cells, and it allows epithelial cells to lose their polarity and cell-to-cell adhesion and transition to mobile and invasive cells. This allows the tumor cells to invade nearby structures and even distant organs (Lamouille, Xu, & Derynck, 2014). Transforming growth factor beta (TGF-β) is an important component of the EMT, and it has been shown to activate fibronectin expression leading to increased cell motility (Ignotz & Massague, 1986).

### 2.5.4 Tumor-stroma ratio

Tumor-stroma ratio (TSR) can be estimated from common hematoxylin-eosin samples by calculating the percentage of tumor cell area compared to the stromal area. It was first discovered as a prognostic factor in colon cancer (Huijbers et al., 2013). Since then it has been shown to have an effect on prognosis in various other types of cancers as well, such as breast cancer, colorectal cancer, cervical cancer, hepatocellular carcinoma, epithelial ovarian cancer, nasopharyngeal cancer, non-small cell lung cancer, endometrial cancer and esophageal adenocarcinoma (Y.
Chen, Zhang, Liu, & Liu, 2015; de Kruijf et al., 2011; Downey et al., 2014; Lv et al., 2015; Panayiotou et al., 2015; J. H. Park, Richards, McMillan, Horgan, & Roxburgh, 2014; J. H. Park et al., 2015; Pongsuvareeyakul et al., 2015; K. Wang et al., 2012; T. Zhang et al., 2015; X. L. Zhang et al., 2014). In these cancer types, low tumor-stroma ratio is typically associated with worsened outcome, compared to cancers with a higher proportion of tumor cells and less extensive stromal compartment. In pancreatic cancer, however, one study showed the opposite result, suggesting that low tumor-stroma ratio associates with improved survival (Bever et al., 2015). As no studies have been made since, the matter requires further research to conclude the usefulness of TSR as a prognostic factor in pancreatic cancer.
3 Aims of the study

The main aim of this study was to characterize the expression of hypoxia markers HIF-1alpha and CAIX and Toll-like receptors TLR2, TLR4 and TLR9 in pancreatic intraepithelial neoplasia and pancreatic ductal adenocarcinoma, and to investigate their impact on patient prognosis. Additionally, the aim was to investigate the impact of stromal components tenascin-C, fibronectin and tumor-stroma ratios on patient prognosis. More specifically, the objectives were:

1. To characterize the expression of TLR2, TLR4 and TLR9 expression in pancreatic intraepithelial neoplasia, pancreatic ductal adenocarcinoma, adjacent normal pancreatic tissue and adjacent pancreatitis, and to investigate the association with patient survival
2. To characterize HIF-1alpha and CAIX expression in pancreatic intraepithelial neoplasia, pancreatic ductal adenocarcinoma, adjacent normal pancreatic tissue and adjacent pancreatitis, and to investigate their association with patient survival
3. To characterize tenascin-C and fibronectin expression in pancreatic cancer and adjacent normal pancreatic tissue, and to investigate their association with patient survival
4. To investigate whether tumor-stroma ratio associates with patient survival in pancreatic ductal adenocarcinoma
4 Materials and methods

4.1 Patients and material

The study material consisting of paraffin-embedded archival specimens of surgically resected PDAC was collected from Oulu University Hospital pathology department archives between the years 1993–2011 (studies I, II and III) and 1993–2015 (Study IV). The final series consisted of 69 patients (studies I, II and III) and 95 patients (study IV) (Table 8). All of the cases were primarily diagnosed with pancreatic ductal adenocarcinoma, and the diagnosis for all cases was confirmed by an expert gastrointestinal pathologist (T.J.K). AJCC 7th edition staging system was applied. Patient clinical data was obtained from patient records and patient survival data from Statistics Finland. The use of the samples and patient data was approved by Oulu University Hospital Ethics Committee and by the National Authority for Medicolegal Affairs (VALVIRA).

4.2 Immunohistochemistry

Dako envision kit (Dako, Copenhagen, Denmark) was used for immunohistochemical detection of the antibody reaction. High-temperature antigen retrieval was done in Tris-EDTA buffer for 15 min. Diaminobenzidine (Dako Basic DAB-kit) was used as chromogen. All of the stainings were done with Dako Autostainer. All of the stainings were performed by highly experienced laboratory technicians. The used antibodies are summarized in Table 9.
Table 8. Summary of the patient clinical data used in the studies.

<table>
<thead>
<tr>
<th>Patient clinical data</th>
<th>I &amp; III</th>
<th>II</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n/N</td>
<td>%</td>
<td>n/N</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;65</td>
<td>34/69</td>
<td>49</td>
<td>34/69</td>
</tr>
<tr>
<td>≥65</td>
<td>35/69</td>
<td>51</td>
<td>35/69</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>36/69</td>
<td>52</td>
<td>36/69</td>
</tr>
<tr>
<td>Female</td>
<td>33/69</td>
<td>48</td>
<td>33/69</td>
</tr>
<tr>
<td>T classification (7th edition)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5/69</td>
<td>7</td>
<td>5/69</td>
</tr>
<tr>
<td>2</td>
<td>23/69</td>
<td>33</td>
<td>23/69</td>
</tr>
<tr>
<td>3</td>
<td>35/69</td>
<td>51</td>
<td>35/69</td>
</tr>
<tr>
<td>4</td>
<td>6/69</td>
<td>9</td>
<td>6/69</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>34/68</td>
<td>50</td>
<td>34/68</td>
</tr>
<tr>
<td>Positive</td>
<td>34/68</td>
<td>50</td>
<td>34/68</td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>14/68</td>
<td>20</td>
<td>18/68</td>
</tr>
<tr>
<td>II</td>
<td>36/68</td>
<td>52</td>
<td>44/68</td>
</tr>
<tr>
<td>III-IV</td>
<td>18/68</td>
<td>26</td>
<td>6/68</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;30mm</td>
<td>23/69</td>
<td>33</td>
<td>23/69</td>
</tr>
<tr>
<td>≥30mm</td>
<td>46/69</td>
<td>67</td>
<td>46/69</td>
</tr>
</tbody>
</table>

Table 9. Used antibodies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Catalogue number</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2</td>
<td>1:50</td>
<td>MAB0066</td>
<td>Abnova, Taipei, Taiwan</td>
</tr>
<tr>
<td>TLR4</td>
<td>1:1000</td>
<td>H000070999-M02</td>
<td>Abnova, Taipei, Taiwan</td>
</tr>
<tr>
<td>TLR9</td>
<td>1:150</td>
<td>IMG-305A</td>
<td>Imgenex, San Diego, CA, USA</td>
</tr>
<tr>
<td>HIF-1alpha</td>
<td>1:300</td>
<td>NB100-105</td>
<td>Novus Biologicals, Littleton, CO, USA</td>
</tr>
<tr>
<td>CAIX</td>
<td>1:100</td>
<td>M75</td>
<td>Dako, Glostrup, Denmark</td>
</tr>
<tr>
<td>Tenascin-C</td>
<td>1:500</td>
<td>610003</td>
<td>Biohit, Finland</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>1:500</td>
<td>NCL-FIB</td>
<td>Leica Biosystems, UK</td>
</tr>
</tbody>
</table>

4.3 Assessment of immunohistochemical stainings

Whole section tissue samples were used in all of the studies. Histological samples were digitalized with Aperio AT2 Console (Leica Biosystems Imaging Inc.,
Nussloch, Germany). Different lesion types were annotated into the digitalized slides by an expert gastrointestinal pathologist (T.J.K). Assessment of the immunohistochemical stainings was done by two independent researchers blinded to the patient clinical data. The intensity of the staining was given a score (0–3) and the extent of the staining was given a percentage (0–100%). Membranous, cytoplasmic and nuclear staining were evaluated separately from all of the samples. A histoscore was calculated by multiplying staining intensity with staining percentage resulting in a score (0–300). Staining for tenascin-C and fibronectin expression in the stroma was evaluated with a 5-point scale as follows: no detectable staining = 0, Focal staining = 1, Areas with diffuse staining present in less than half of stromal area = 2, Expression distributed in more than half but not in all parts of the tumor stroma = 3, Expression extending throughout the stroma = 4. When inter-observer difference was less than one point in intensity and 30% in percentage, the mean value of the independent estimates was used for statistical analysis. Cases with higher differences were re-evaluated and a single score was given by consensus between the two observers.

The site of evaluation for tumor tissue was chosen from the most representable area. All of the samples contained invasive front. PanIN-lesions were chosen further away from the cancerous tissue. For each study sample, one of each lesion (PanIN, carcinoma, pancreatitis and exocrine pancreas) was evaluated. All of the study samples did not contain PanIN-lesions. The stroma was evaluated adjacent to the tumor tissue to represent tumor-associated stromal response (Figure 3).
4.4 Local inflammatory response

The inflammatory cell response in the invasive front of the cancer and in the bulk of the cancer was evaluated according to guidelines described earlier by Laurila et al. (Laurila et al., 2005). The response was evaluated by two independent observers. The response was given a score ranging from absent (score 0) to strong increase (score 3). The mean value of two independent estimates was then used to divide the cohort into three equal groups (low, moderate and high).
4.5 Tumor-stroma ratio

Tumor/stroma ratio was evaluated from hematoxylin and eosin-stained sections. Tumor-stroma ratio was calculated from whole section samples. The microscopic field of view (10x) was positioned so that all four corners contained tumor cells. The tumor-stroma ratio was evaluated separately from the tumor bulk and invasive front. Percentage (0–100%) of tumor cell area vs stromal area in representative microscope view was assessed, referred to as tumor/stroma ratio. Mean value of two independent researchers was used with no need for re-evaluation, as the inter-observer difference was less than 30%. Significance of tumor/stroma percentage was analyzed by using two groups (≤35% and >35%) divided according to the median value.

4.6 Statistical analysis

For statistical analysis, IBM SPSS 22.0 (IBM corp., Armonk, NY) was used. The chi-square test was used to calculate statistically significant differences between protein expression and clinicopathological variables. Spearman’s two-tailed correlation test was used to calculate correlations between different proteins. Wilcoxon paired samples test and Kruskal-Wallis were used to compare protein expression between different lesions. Kaplan-Meier was used to calculate life tables.
and survival curves were compared with the log-rank test. Cox proportional hazards model was used in the multivariate analysis. Interobserver agreement was analyzed using Cohen’s kappa value.
5 Results

5.1 TLR expression in pancreatic lesions (Studies I and III)

All of the studied TLRs were expressed in the pancreas. Positive expression was found in normal and inflamed ductal epithelium, adjacent normal pancreatic tissue and pancreatitis, in the precursor lesions PanINs, as well as in pancreatic ductal adenocarcinoma. TLR expression in the pancreas was mainly membranous or cytoplasmic, and positive nuclear staining was only occasionally visible (Figure 5).

The strongest cytoplasmic TLR2 expression was seen in PanIN3 lesions, and TLR2 expression increased linearly from PanIN1 lesions towards PanIN3 lesions. TLR2 expression in all of the PanIN lesions was significantly stronger than in normal ductal epithelium. TLR2 expression in the cancer was significantly stronger compared to adjacent normal pancreatic ducts and exocrine pancreas. TLR2 expression was equally strong in PanINs and cancer.

The strongest TLR4 expression was seen in the pancreatitis ducts. All of the PanIN lesions showed high levels of TLR4 expression; however, there was no difference in expression levels between PanIN lesion and adjacent normal pancreatic ducts. TLR4 was strongly expressed in pancreatic cancer, and the expression was significantly stronger than in adjacent normal exocrine pancreas, but did not differ from normal pancreatic ducts.

TLR9 was also expressed in the pancreatic cancer as well as in the adjacent normal pancreatic tissue. TLR9 expression was significantly stronger in cancer cells compared to normal pancreatic ducts, but did not differ from normal acinar cells. PanIN1 lesions showed strongest TLR9 expression, and in all of the PanIN lesions, TLR9 expression was significantly stronger than in adjacent normal pancreatic ducts.
Fig. 5. Examples of different TLR staining patterns taken from our own material. a) Pancreatic ductal adenocarcinoma with prominent TLR2 expression, b) TLR4 expression, and c) TLR9 expression.
5.2 Hypoxia markers in pancreatic lesions (Studies II and III)

HIF-1alpha and CAIX were both expressed in all of the studied lesions (Figure 6). HIF-1alpha expression was mainly nuclear, and occasional cytoplasmic staining was seen. CAIX was expressed in the membrane of the cells.

Strongest nuclear HIF-1alpha staining was seen in PanIN3 lesions, and HIF-1alpha expression increased linearly from PanIN1 to PanIN3. In PanIN3 lesions, HIF-1alpha expression was significantly stronger than in the adjacent normal pancreatic ducts. In pancreatic cancer, HIF-1alpha was also abundantly expressed, and the expression was significantly stronger than in normal pancreatic ducts and adjacent normal exocrine pancreas.

CAIX expression increased linearly towards higher grade PanIN, PanIN3 – lesions showing the strongest membranous CAIX expression. In PanIN2 and PanIN3 lesions, the expression was significantly stronger than in adjacent normal pancreatic ducts. CAIX expression in PanIN3 was significantly stronger than in cancer cells. CAIX in cancer cells did not differ from normal pancreatic ducts, but was significantly stronger compared to normal exocrine pancreas.

We found no correlation between nuclear HIF-1alpha and membranous CAIX expression.

5.3 Tenascin-C and fibronectin in pancreatic cancer (Study IV)

Tenascin-C and fibronectin expression were assessed separately from the invasive front of the tumor and from the bulk of the tumor. Both tenascin-C and fibronectin expression was limited to the stromal component of the tumor, and epithelial cells showed no positive tenascin-C or fibronectin expression (Figure 7). Furthermore, adjacent normal pancreatic tissue showed negative staining for tenascin-C and fibronectin. Tenascin-C was expressed abundantly both in the invasive front and bulk of the tumor. There was no difference in expression levels between these two locations. Fibronectin was also expressed in the majority of the cases. Fibronectin was equally expressed in the invasive front and bulk of the tumor.
Fig. 6. Examples of a typical staining pattern for hypoxia markers in pancreatic ductal adenocarcinoma. Images taken from our material. a) Pancreatic ductal adenocarcinoma with moderate to strong nuclear HIF-1alpha expression in the majority of cancer cells. b) Pancreatic ductal adenocarcinoma with prominent membranous CAIX expression.
Fig. 7. Examples of tenascin-C and fibronectin staining patterns taken from our own material. a) Prominent tenascin-C expression. The expression pattern is restricted to the stroma, and tumor cells are negative for tenascin-C. b) Fibronectin expression restricted to the stroma with negative tumor cells.
5.4 Correlation with clinicopathological variables (Studies I, II, III and IV)

All of the markers were assessed separately to find associations between markers and clinicopathological variables.

High cytoplasmic TLR4 expression associated with higher tumor grade while low membranous TLR9 associated with low BMI. Higher than median (>7.3 x 10^9/l) leukocyte count from preoperative blood samples associated with low cytoplasmic TLR9 expression of the tumor. There were no other associations between TLRs and clinicopathological variables.

The only association of hypoxia markers with clinicopathological variables was between membranous CAIX and sex. Males showed weaker membranous CAIX expression compared to females. Fibronectin expression of the tumor bulk associated with high T-class. No other associations were found between stromal markers and clinicopathological variables. All of these findings are summarized in Table 10.

5.5 TLRs and association with local inflammatory response (Study I)

We tested TLR2, TLR4 and TLR9 expression and association with local inflammatory response. Low membranous TLR2 expression associated with low local inflammatory response in the tumor bulk. No other associations were found between local inflammatory response and TLR expression.

5.6 Association between TLR expression and hypoxia (Study III)

TLR expression and HIF-1alpha and CAIX expression were tested for correlation in all of the studied lesions. HIF-1alpha correlated with nuclear TLR9 expression in normal pancreatic ducts and with nuclear TLR2 in inflamed ducts. Within the PanIN1-group, nuclear HIF-1alpha correlated with TLR2 expression, and in the PanIN2-group HIF-1alpha correlated with nuclear TLR9 expression. We found no statistically significant correlation between CAIX and any of the studied TLRs. There were no significant correlations between HIF-1alpha and TLRs in pancreatic cancer tissue, either.
5.7 Patient survival (Studies I, II, III and IV)

TLRs, hypoxia markers and stromal markers were studied to find their impact on patient prognosis. Furthermore, clinicopathological variables were tested for effect on patient survival (Table 10).

Tumor size, lymph node metastases, tumor stage, sex or age were not associated with patient survival in our material.

High cytoplasmic TLR9 expression associated with improved survival times in univariate and multivariate analysis. No other TLR had impact on patient survival. Weak nuclear HIF-1alpha associated with poor prognosis in both univariate and multivariate analysis. Tenascin-C or fibronectin had no impact on patient survival in the whole group analysis. In a subgroup analysis containing T1 and T2 class tumors, high tenascin-C of the tumor bulk associated with poor prognosis. Tumor-stroma ratio did not associate with patient survival.
Table 10. The expression of studied markers in relation to clinicopathological variables and patient survival. ¹NS, No significant correlation. *T1/T2 tumors

<table>
<thead>
<tr>
<th>Marker</th>
<th>T-class</th>
<th>Grade</th>
<th>Sex</th>
<th>BMI</th>
<th>local inflammatory response</th>
<th>Leukocytes</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2</td>
<td>NS¹</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>Low expression and low response, p=0.019</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>TLR4</td>
<td>NS</td>
<td>High expression and high grade, p=0.010</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>TLR9</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>Low expression and low BMI, p=0.031</td>
<td>Low expression and high leukocyte count, p=0.031</td>
<td>NS</td>
</tr>
<tr>
<td>HIF-1alpha</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>High expression and improved survival, p=0.001</td>
</tr>
<tr>
<td>CAIX</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>Low expression and male sex, p=0.038</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Tenascin-C</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>High</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

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6 Discussion

In this study, the expression of TLR2, TLR4, TLR9, HIF-1alpha, CAIX, tenascin-C and fibronectin was evaluated in pancreatic ductal adenocarcinoma and in precursor lesions PanINs, as well as in the adjacent normal pancreatic tissue and pancreatitis. Furthermore, we evaluated their association with clinicopathological variables and investigated their impact on patient prognosis. TLRs, HIF-1alpha, CAIX, tenascin-C and fibronectin were abundantly expressed in pancreatic ductal adenocarcinoma. Adjacent normal pancreatic tissue also showed varying levels of TLR, HIF-1alpha and CAIX expression; however, tenascin-C and fibronectin were not found in normal pancreatic tissue. High TLR9 expression associated with improved survival of the patients, and interestingly, weak HIF-1alpha expression marked poor prognosis. In T1 and T2 tumors, high tenascin-C expression indicated poor prognosis; however, this effect was not observed in the whole material. Furthermore, in our material, tumor-stroma ratio was not associated with patient survival.

6.1 TLRs in pancreatic intraepithelial neoplasia

All of the TLRs were abundantly expressed in pancreatic intraepithelial neoplasia, and TLR2 showed a linear increase from PanIN1 lesions towards PanIN3 lesions. In esophageal adenocarcinoma and its precursor lesions, TLR expression levels are elevated, suggesting their role in the development of the cancer at earlier stages (Helminen et al., 2016; Huhta et al., 2015). We found no previous studies investigating TLR in pancreatic intraepithelial neoplasia, and therefore we concluded for the first time TLR expression in pancreatic intraepithelial neoplasia. TLR2 and TLR9 were more abundantly expressed in PanIN lesions compared to adjacent normal pancreatic ducts. These suggest that PanIN lesions differ from normal pancreatic ducts in terms of inflammatory response. Recent studies have suggested that inflammatory responses could already be activated in PanIN2 lesions (Moniaux et al., 2008; Saloman et al., 2016). Our material consisted only of PanIN accompanied with invasive pancreatic cancer, and this could have some effect on the microenvironment and inflammatory response within the studied PanIN lesions. However, our group has previously concluded that pancreatic cancer has no effect on TLR expression in adjacent normal exocrine pancreas, which suggests that there is only limited field effect in terms of TLR expression in pancreatic cancer (Huhta, Helminen, Kauppila et al., 2016). Therefore, it is possible
that the abnormal inflammatory response and TLR dysregulation could be the driving force in pancreatic intraepithelial neoplasia and early stage pancreatic cancer.

6.2 TLRs in pancreatic ductal adenocarcinoma

TLR2, TLR4 and TLR9 were all expressed in pancreatic ductal adenocarcinoma. Previous studies have concluded that TLR2, TLR4 and TLR9 are found in pancreatic cancer, and TLR2 and TLR4 have been suggested to have impact on patient prognosis (Lanki et al., 2018; Sun et al., 2016; Vaz & Andersson, 2014; Wu et al., 2011; Zambirinis et al., 2015; J. J. Zhang et al., 2010). TLR2 and TLR4 were not associated with patient survival in our material. Low membranous TLR2 expression associated with low local inflammatory response of the tumor. High cytoplasmic TLR4 expression was associated with higher tumor grade, but no other associations between clinicopathological factors and TLRs were found in our material.

In TLR9, previous results are limited, and none of the previous results confirm its significance in patient prognosis. In our material, high TLR9 expression indicated improved prognosis, while low TLR9 was an independent prognostic factor for poor prognosis. TLR9 was expressed equally in adjacent normal pancreatic tissue and pancreatic cancer, and no statistical difference was found between the expression levels of these two. The explanation for our finding remains speculative. Our group recently showed that in germ-free mice, TLR9 was widely expressed in normal pancreatic tissue, and the expression levels were significantly higher than in conventional mice (Huhta et al., 2016). It has also been suggested that bacteria could have down-regulatory effects on TLRs (Otte, Cario, & Podolsky, 2004). Recent epidemiological studies have shown that oral pathogens, capable of reaching the pancreas through the biliary tract or bloodstream, are associated with risk of pancreatic cancer, and it has been suggested that bacterial exposure could play a role in pancreatic cancer progression (Michaud & Izard, 2014; C. Wang & Li, 2015; Zambirinis, Pushalkar, Saxena, & Miller, 2014). Taking these results together, bacterial exposure could be responsible for the different TLR9 levels in pancreatic cancer, and low levels of TLR9 could be a sign of prolonged bacterial exposure. Another possible explanation is that cancer developed through prolonged bacterial exposure could have different properties and therefore different tendencies to grow and invade compared to cancers developed through other ways (alcohol use, chronic pancreatitis etc.). These explanations remain hypothetical, but
in the future, modern methods recognizing bacterial presence in pancreatic tissue might shed more light on this matter.

Another possible explanation for our result could be that cancers with low TLR9 levels could evade the immune system, thus allowing for more rapid tumor growth and invasion. Lack of signals to adaptive immunity through TLR9 could lead to insufficient immune response towards the tumor cells.

In a study by Tuomela et al. the group discovered that TLR9 expression had opposite effect on patient survival depending on the level of hypoxia in breast cancer. They found out that under hypoxic conditions, low TLR9 expression associated with increased invasion of the tumor cells (Sandholm et al., 2014). They further hypothesized that this could be the case in other hypoxic cancer types as well, namely renal carcinoma and pancreatic cancer. Furthermore, they showed that HIF-1alpha activates TLR9 expression in breast cancer cells. As we found no correlation between hypoxia markers and TLRs, no conclusions can be drawn from our studies. However, in the future this could be an interesting field of research to investigate whether under hypoxic conditions, altering TLR9 expression levels could induce invasive properties of the tumor cells in pancreatic cancer as well.

6.3 Hypoxia and pancreatic ductal adenocarcinoma

HIF-1alpha and CAIX were abundantly expressed in pancreatic cancer. CAIX expression did not correlate with HIF-1alpha in cancer lesions. Interestingly, weak HIF-1alpha was associated with poor prognosis. HIF-1alpha and CAIX were expressed in PanIN lesions, and HIF-1alpha showed a linear increase from PanIN1 towards PanIN3.

It is yet unclear at which point the hypoxic microenvironment develops in pancreatic cancer. Typically, it has been thought that it is mostly the growing stroma that contributes to the formation of the hypoxic microenvironment in pancreatic cancer. Previous studies suggested that PanIN lesions show elevated HIF-1alpha levels and could therefore be hypoxic (Koong et al., 2000; K. E. Lee et al., 2016). This sounds plausible, as hypoxia is a known factor for selection of more malignant cells, and tumorigenesis could therefore be driven by the early emergence of hypoxia already from the early stages. Our findings are in line with these previous results; however, some aspects must be taken into account while interpreting the results from our study. Our material consisted of cases where pancreatic cancer had already developed, and we therefore believe that this could have a dramatic effect on the microenvironment and oxygen levels within the pancreatic tissue. It is
possible that the elevation in hypoxia markers observed in our material could be secondary to the tumor growth. To minimize this effect, the studied PanIN lesions were chosen further away from the primary tumor.

A meta-analysis by Ye et al. suggests that high HIF-1alpha expression in pancreatic cancer is associated with poor prognosis, a result that is contradictory to ours (Ye et al., 2014). This meta-analysis consisted mainly of studies on eastern populations, and current evidence of HIF-1alpha expression and survival is lacking in western populations. Furthermore, some methodological differences can be found in the studies included in the meta-analysis. Some of the studies included cytoplasmic rather than nuclear HIF-1alpha staining in the analysis. HIF-1alpha is biologically active in the nucleus, and therefore it seems reasonable to include only the nuclear staining in the analysis (Semenza, 1999). Furthermore, the use of negative control was not always reported in the original articles. This is somewhat relevant, as endogenous biotin is known to cause false positive results in immunohistochemical stainings, and the use of biotin-based methods should contain negative control stainings (Bussolati, Gugliotta, Volante, Pace, & Papotti, 1997; Duhamel & Johnson, 1985; Vosse, Seelentag, Bachmann, Bosman, & Yan, 2007). In our material, all the stainings were performed by experienced laboratory technicians and negative control was routinely applied.

The discordant results can to some extent be explained by these previous aspects, but they are unlikely to explain the result completely. In addition to hypoxia, HIF-1alpha can also be upregulated by other factors, such as alterations in oncogenes and tumor suppressor genes (Fillies et al., 2005; Kamisawa, Wood, Itoi, & Takaori, 2016b; Laughner, Taghavi, Chiles, Mahon, & Semenza, 2001; Maxwell et al., 1999; Ravi et al., 2000; Semenza, 2003; Zhong et al., 2000). HIF-1alpha expression, independent of oxygen levels, results in a diffuse staining pattern seen in breast and oropharyngeal cancers (Aebersold et al., 2001; Bos et al., 2003; Kuijper, van der Groep, van der Wall, & van Diest, 2005). As we found no correlation between HIF-1alpha and another hypoxia marker, CAIX, there is a possibility that the HIF-1alpha expression seen in PanINs and carcinoma in our material could be a result of various other processes. Therefore, we cannot reliably determine whether the HIF-1alpha expression seen in PanINs represents true lack of oxygen, and hence the emergence of hypoxic microenvironment in earlier stages warrants additional evidence.
6.4 Tumor microenvironment in pancreatic ductal adenocarcinoma

Tenascin-C and fibronectin were abundantly expressed in the stroma of pancreatic cancer. In adjacent normal pancreatic tissue, there was no positive staining for tenascin-C or fibronectin. There were no significant differences in expression levels between the invasive front and bulk of the tumor. No positive correlation was found between tenascin-C and fibronectin and tumor-stroma ratio, suggesting that tenascin-C and fibronectin expression are independent of the extent of the stroma. Tenascin-C, fibronectin or tumor-stroma ratio had no impact on patient survival in the whole material, however in T1 and T2 tumors high tenascin-C associated with poor prognosis.

Abundant stroma leads to hypovascularization, poor delivery of nutrients and chemoresistance in solid tumors (Topalovski & Brekken, 2016). Therefore, the role of stroma in cancer survival has been under rigorous research in recent years. Tumor-stroma ratio is a relatively simple and easily replicable method of assessing the extent of the stroma in solid tumors, and a multitude of studies investigating its usefulness as a prognostic factor in various cancers have been conducted. Typically,
a low tumor-stroma ratio is linked to poor prognosis in cancers. This means that there are fewer tumor islets in between the vast stroma. In pancreatic cancer, however, the matter appears more complex. Only one previous study investigated the tumor-stroma ratio as a prognostic factor in pancreatic cancer (Bever et al., 2015). In that study, Bever et al. concluded that a high tumor-stroma ratio associated with poor prognosis, a result contradictory to other cancer types. The authors hypothesized that the result could be due to slow-growing pancreatic cancer accumulating more fibrotic stroma because of a longer-lasting inflammatory response. Another possibility is that as stroma-rich tumors typically have more differentiated histology than stroma-poor tumors, this could partly explain their surprising results (Rhim et al., 2014). However, in our material we found no significant difference in survival times between stroma-rich and stroma-poor cancers, suggesting that although tumor-stroma ratio may be useful in other solid malignancies, in pancreatic cancer, a cancer type typically associated with abundant desmoplastic stroma, the value of tumor-stroma ratio as a prognostic factor could be more modest.

Tenascin-C and fibronectin are glycoproteins of the extracellular matrix involved in many normal functions in healthy organisms (Erickson, 1993; Pankov & Yamada, 2002). In cancer, tenascin-C contributes to many features vital to cancer progression, such as cancer cell proliferation, cell migration and invasion and angiogenesis (Thakur & Mishra, 2016; Yoshida, Akatsuka, & Imanaka-Yoshida, 2015). Fibronectin is equally involved in cell invasion, metastasis and angiogenesis during carcinogenesis (Akiyama et al., 1995; Van Obberghen-Schilling et al., 2011). Previous studies have linked tenascin-C and fibronectin to poor prognosis in various malignancies. Typically, high levels of tenascin-C or fibronectin indicate an adverse prognosis (Brunner et al., 2004; Lundin et al., 2007; Ohno et al., 2008; Wiksten et al., 2003; S. L. Yang, Ren, Wen, & Hu, 2016). In pancreatic cancer, tenascin-C itself does not seem to associate with survival, but with co-expression with MMP-9 there was an association to poor prognosis (Xu et al., 2015). In our material, in less advanced T-class tumors, high tenascin-C associated with poor prognosis. This suggests that at earlier stage, tenascin-C contributes to survival, but as the tumor progresses the effect disappears. This is biologically reasonable as a majority of pancreatic cancer tumors have abundant stroma, which may itself contribute to the prognosis.
6.5 Tenascin-C and inflammation

Tenascin-C is involved in tissue injury and cellular stress, and upregulated in inflammation and tissue remodeling. Tenascin-C functions both in physiological tissue repair as well as in pathological inflammation and fibrosis. The mechanism by which tenascin-C induces inflammatory effects is currently unclear, but some targets have been identified (Marzeda & Midwood, 2018). One of these possible targets is TLR4, as research from previous years has shown that tenascin-C functions as an activator of TLR4. The complete signaling cascade involving tenascin-C and TLR4 is, however, currently unclear (Zuliani-Alvarez et al., 2017). In rheumatoid arthritis, tenascin-C/TLR4 interplay was found, suggesting a role as a possible regulator of persistent inflammatory response (Midwood et al., 2009). In hepatocellular carcinoma, it was discovered that tenascin-C/TLR4 signaling is a key part of the development of hepatocellular carcinoma (Benbow et al., 2016). In other cancers, this matter is less known as evidence is currently scarce. In the future, it would be interesting to know whether there is an interplay between tenascin-C and TLR4 or other TLRs in various other cancer types, especially pancreatic cancer, where chronic and persistent inflammatory stroma is evident.

6.6 Limitations and strengths of this study

The limitations to this study include the relatively small study population (n=69 and n=95), and the age of the material. The study sample size in other studies investigating hypoxia markers and Toll-like receptors as prognostic factors in pancreatic cancer vary from n=39 to n=150, the average being somewhere around 90-100 (Lanki et al., 2018; Ye et al., 2014). However, the material used was collected from one geographical area from a single hospital with no apparent selection bias. All of the samples were diagnosed as pancreatic ductal adenocarcinoma, and the diagnosis was confirmed by an expert gastrointestinal pathologist. Another shortcoming of this study is the varying number of cancer lesions in different immunohistochemical stainings. This is due to some of the paraffin blocks running out of cancerous tissue. This decreased the number of cases in some of the stainings. The follow-up data was acquired from Statistics Finland. This adds to the reliability of the study as the date and cause of death are reliably recorded in Finland. The determination of R0/R1 resection may be unreliable in the older study samples, as it has become part of routine diagnostics only in the recent years, and may not have been reported in a sufficient way in the older samples.
The use of immunohistochemistry as the only method of evaluating protein expression also has some limitations. However, the immunohistochemical stainings were consistent and made according to routine protocols by experienced laboratory technicians, increasing the reliability. The evaluation of the immunohistochemical stainings was made by two independent researchers, blinded to the patient clinical data, minimizing any bias. The evaluation was made according to guidelines used in various previous studies. The inter-observer agreement was excellent as only few of the cases needed re-evaluation.

The normal pancreatic tissue that was evaluated was adjacent to the tumor, and this could have some effect on the biology of the tissue. Therefore, it should be noted that normal healthy pancreas could differ from the adjacent normal pancreatic tissue observed in our studies. None of the patients received preoperative adjuvant therapy. In the future, the increased use of neoadjuvant therapy may have some effect on the results of prognostic markers, as it may change the conditions within the tumor dramatically.

6.7 Future aspects

We found that high TLR9 expression indicated improved survival in pancreatic cancer. One possible, yet hypothetical, explanation for this could be different bacterial exposure between the low and high TLR9 groups. In the future, it would be interesting to investigate possible bacterial presence using modern 16s ribosomal RNA-based methods. If bacterial material was present and association with decreased TLR9 levels was found, it would open a completely new field of research. It would also support our hypothesis of the importance of bacterial exposure in pancreatic cancer progression.

Since the studied pancreatitis lesions were analyzed from tissue samples containing carcinoma cells, it is impossible to determine whether the pancreatitis detected in the samples is due to tumor growth or has been there as a carcinogenic factor long before the emergence of the cancer. In the future, it would be interesting to investigate whether pancreatitis without cancer has similar features in terms of TLR expression. As pancreatitis is characterized by strong stromal and inflammatory response, it would be of interest to investigate stromal response markers tenascin-C and fibronectin as well as TLR and the correlation between these markers in material consisting only of chronic pancreatitis patients.

Since there is only preliminary evidence of tenascin-C/TLR interplay in cancers, it would be reasonable to investigate this matter further in pancreatic
cancer. As it seems evident that pancreatic cancer is characterized by abundant stroma as well as tenascin-C expression, it would be natural to test the hypothesis of tenascin-C as a regulator for TLR-mediated inflammatory responses in pancreatic cancer.

Furthermore, it would be interesting to investigate whether the stroma of chronic pancreatitis differs from stroma of cancerous origin in terms of stromal marker expression.

As in the prognostic factor research nowadays, our results call for validation studies to establish the role of each marker.
7 Summary and conclusions

In conclusion, we showed that TLRs 2, 4 and 9 are widely expressed in pancreatic intraepithelial neoplasia and in pancreatic ductal adenocarcinoma, and TLR9 associates with prognosis. HIF-1alpha, CAIX, tenascin-C and fibronectin are also expressed in pancreatic ductal adenocarcinoma, and HIF-1alpha associates with prognosis. More specifically, the conclusions are:

1. TLR2, TLR4 and TLR9 are expressed in pancreatic intraepithelial neoplasia and in pancreatic ductal adenocarcinoma as well as in adjacent normal pancreatic tissue and pancreatitis. High TLR9 expression is associated with improved prognosis in pancreatic ductal adenocarcinoma.

2. HIF-1alpha and CAIX are expressed in pancreatic intraepithelial neoplasia and pancreatic ductal adenocarcinoma as well as in the adjacent normal pancreatic tissue and adjacent pancreatitis. Weak HIF-1alpha is associated with poor prognosis in pancreatic ductal adenocarcinoma.

3. Tenascin-C and fibronectin are expressed in the stroma of the pancreatic ductal adenocarcinoma, and absent from adjacent normal pancreatic tissue. Neither tenascin-C or fibronectin had impact on prognosis in the whole material, but in T1 and T2 tumors, high tenascin-C expression associated with poor prognosis.

4. Tumor-stroma ratio did not associate with prognosis in our material.
References


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Original publications


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