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PROGNOSTIC VALUE OF INFLAMMATION-RELATED BIOMARKERS IN GASTRIC CANCER

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ACADEMIC DISSERTATION

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*These authors contributed equally to these works.

The original publications are referred to in the text by their Roman numerals (I–IV).
2 ABSTRACT

Gastric cancer is the second most common cause of cancer-related mortality worldwide. The poor prognosis largely results from late diagnosis, often occurring when disease is already at an advanced stage. At the time of diagnosis, treatment with curative intent is possible for less than half of all patients. Currently, the only curative treatment is radical surgery. Despite the decreased incidence of gastric cancer in developed countries given changes in lifestyle and the treatment of Helicobacter pylori (H. pylori) infection, prognosis remains poor. More precise knowledge regarding the underlying pathophysiology is needed in order to allow for earlier diagnosis and to improve prognosis.

Chronic inflammation of the gastrointestinal tract mucosa predisposes individuals to cancer with several proteins contributing to the crosstalk between inflammation and cancer. Matrix metalloproteinases (MMPs) can promote cancer cell invasion and metastasis by degrading the extracellular matrix. In previous studies, a high expression of matrix metalloproteinase 14 (MMP14) associated with metastasized gastric cancer and a poor outcome. Prospero homeobox protein 1 (PROX1) is a transcription factor functioning in cell fate determination and organ development, and also expressing in various cancers. PROX1 acts context dependently either as an oncogene or as a tumor suppressor. PROX1 was recently shown to inhibit the transcription of MMP14 in several cancers, although it was not examined in gastric cancer. Toll-like receptors (TLRs) are pattern recognition receptors essential to innate immunity. They express in malignant diseases, activated by damage-associated molecular patterns. Tumor-associated trypsin-2 (TAT-2) and its inhibitor, tumor-associated trypsin inhibitor (TATI), can promote carcinogenesis by activating endothelial growth factor receptors, pro-urokinase, and MMPs. In addition, C-reactive protein (CRP) is a well-known and widely used inflammatory biomarker. Elevated preoperative CRP levels have been attributed to a poor outcome in colonic, pancreatic, and gastric cancers. Therefore, this study aimed to evaluate the significance of these potential prognostic biomarkers in gastric cancer.

The cohort consisted of 313 individuals operated on between 2000 and 2009 for histologically confirmed gastric adenocarcinoma in the Department of Surgery, Helsinki University Hospital, Finland. Preoperative blood samples were collected from 240 patients with gastric cancer and from 48 control patients with benign disease. Tissue MMP14, PROX1, TLR1, TLR2, TLR4, TLR5, TLR7, and TLR9 expression levels were studied using immunohistochemistry. Soluble serum MMP14
levels were determined using an enzyme-linked immunosorbent assay. Serum TAT-2, TATI, and plasma CRP levels were determined using time-resolved immunofluorometric assays.

A high MMP14 expression, whether in tissue samples or serum, predicted a poor outcome and a high serum MMP14 remained an independent prognostic factor in the multivariate survival analysis. A high tissue MMP14 predicted an unfavorable outcome, particularly among those with a low PROX1 level. Furthermore, a high tissue TLR5 expression predicted a better outcome. We also found that TLR1, TLR2, TLR4, TLR7, and TLR9 did not serve as prognostic biomarkers across the entire cohort. Nevertheless, TLR9 served as a prognostic factor among those with stage II disease and TLR7 among those with stage III disease. High serum TAT-2 and TATI both identified patients with poor prognoses, with TATI remaining significant in the multivariate survival analysis. Moreover, serum TAT-2 levels were higher among patients with gastric cancer than among controls. Interestingly, preoperative CRP did not serve as a prognostic biomarker in this cohort of gastric cancer patients.

None of the biomarkers studied are currently in routine clinical use in gastric cancer. However, this thesis shows that several of the biomarkers examined hold potential as prognostic factors in gastric cancer and represent promising candidates for further investigation. In conclusion, survival seems worse among gastric cancer patients with a high tissue MMP14 expression, particularly with concurrent low PROX1 levels. Thus, a high serum MMP14 may serve as an independent unfavorable prognostic biomarker. Survival appears better among patients with a high tissue TLR5 expression. Additionally, high tissue TLR7 and TLR9 expressions appear to serve as favorable prognostic biomarkers in certain subgroups. In addition, a high serum TATI may serve as an independent unfavorable prognostic factor. Lastly, serum TAT-2 levels appear higher among patients with gastric cancer than among controls and a high TAT-2 may serve as a marker of an unfavorable prognosis.
3 TIIVISTELMÄ (FINNISH ABSTRACT)


4 Abbreviations

AJCC  American Joint Committee on Cancer
APC  Adenomatous polyposis coli
Bel-2  B-cell lymphoma 2 protein
BSA  Bovine serum albumin
CA  Carbohydrate antigen
CagA  Cytotoxin-associated gene A
CDH1  E-cadherin coding gene
CEA  Carcinoembryonic antigen
CI  Confidence interval
CIN  Chromosomal instability
COX-2  Cyclooxygenase-2
CRP  C-reactive protein
CSF-1  Colony stimulating factor 1
CTL  Cytotoxic T cell
CTLA4  Cytotoxic T lymphocyte-associated protein 4
DAMP  Damage-associated molecular pattern
DC  Dendritic cell
DNA  Deoxyribonucleic acid
EBV  Epstein–Barr virus
E-cadherin  Epithelial cadherin
ECM  Extracellular matrix
EGD  Esophagogastroduodenoscopy
EGF  Epidermal growth factor
EGFR  Epidermal growth factor receptor
ELISA  Enzyme-linked immunosorbent assay
EMT  Epithelial–mesenchymal transition
FAP  Familial adenomatous polyposis
GAPPS  Gastric adenocarcinoma and proximal polyposis of the stomach
GEJ  Gastroesophageal junction
GPS  Glasgow prognostic score
GS  Genetic stability
*H. pylori*  *Helicobacter pylori*
HCl  Hydrochloric acid
HDGC  Hereditary diffuse gastric cancer
HER2  Human epidermal growth factor receptor 2
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<tr>
<td>HR</td>
<td>Hazard ratio</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>IQR</td>
<td>Interquartile range</td>
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<td>mAb</td>
<td>Monoclonal antibody</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>MDSC</td>
<td>Myeloid-derived suppressor cell</td>
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<td>mGPS</td>
<td>Modified Glasgow prognostic score</td>
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<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<td>MMP14</td>
<td>Matrix metalloproteinase 14</td>
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<td>MSI</td>
<td>Microsatellite instability</td>
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<td>MTI-MMP</td>
<td>Membrane type 1 matrix metalloproteinase</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
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<tr>
<td>pAb</td>
<td>Polyclonal antibody</td>
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<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PD1</td>
<td>Programmed cell death 1</td>
</tr>
<tr>
<td>PDL</td>
<td>Programmed cell death ligand</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<td>PGE2</td>
<td>Prostaglandin E2</td>
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<td>pRb</td>
<td>Retinoblastoma protein</td>
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<td>PROX1</td>
<td>Prospero homeobox protein 1</td>
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<td>PSTI</td>
<td>Pancreatic secretory trypsin inhibitor</td>
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<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>ROC</td>
<td>Receiver operating characteristic</td>
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<tr>
<td>RT-PCR</td>
<td>Real-time polymerase chain reaction</td>
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<tr>
<td>SEM</td>
<td>Self-expanding metallic stent</td>
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<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>SMT</td>
<td>Somatic mutation theory</td>
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<td>SPINK1</td>
<td>Serine protease inhibitor Kazal-type 1</td>
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<tr>
<td>TAM</td>
<td>Tumor-associated macrophage</td>
</tr>
<tr>
<td>TAN</td>
<td>Tumor-associated neutrophil</td>
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<tr>
<td>TAT-2</td>
<td>Tumor-associated trypsin 2</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>TATI</td>
<td>Tumor-associated trypsin inhibitor</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
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<tr>
<td>Th1</td>
<td>T-helper type 1 cell</td>
</tr>
<tr>
<td>Th2</td>
<td>T-helper type 2 cell</td>
</tr>
<tr>
<td>Th17</td>
<td>T-helper type 17 cell</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinase</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TMA</td>
<td>Tissue microarray</td>
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<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
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<td>TNM</td>
<td>Tumor, Node, Metastasis</td>
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<tr>
<td>TR-IFMA</td>
<td>Time-resolved immunofluorometric assay</td>
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<tr>
<td>VacA</td>
<td>Vacuolating cytotoxin A</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>WHO</td>
<td>World Health Organization</td>
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5 INTRODUCTION

Globally, gastric cancer stands as the sixth most common cancer, only surpassed in incidence by skin, colon, prostate, breast, and lung cancers (Bray et al. 2018). In Finland, gastric cancer is the twelfth most common cancer among men and the fourteenth most common cancer among women (Finnish Cancer Registry 2019). In recent decades, the incidence of gastric cancer has decreased, possibly due to the decreasing prevalence of Helicobacter pylori (H. pylori) infections and given changes in lifestyle factors including decreasing rates of smoking (Ang and Fock 2014; Sitarz et al. 2018). Despite declining incidence rates and improvements in treatment, however, prognosis remains poor. Gastric cancer represents the second most common cause of cancer-related mortality globally. This poor prognosis largely stems from a late diagnosis. Stage I gastric cancer features a favorable prognosis with 5-year survival of over 90%, whereas for stage IV gastric cancer 5-year survival falls to under 10% even in high-quality centers (Kim et al. 2017). Early stage gastric cancer is often symptomless, while symptoms are associated with an advanced stage. Today, curative treatment of gastric cancer always requires radical surgery. Thus, the development of novel therapies and identifying biomarkers remain essential to achieving a better prognosis in gastric cancer.

Traditionally, gastric cancer was classified according to its histological features without taking into account its molecular profile. Genetic and epigenetic alterations in gastric cancer are diverse, while novel subtyping is crucial for further advances in treatment (Choi et al. 2016; Shimizu et al. 2018). The Cancer Genome Atlas Research Network (2014) recently proposed a novel molecular subtyping system, although this is not yet in clinical use.

Cancer cells are derived from normal cells by acquiring cumulative procancerous mutations and breaking away from the proliferation controlling mechanisms, ultimately reaching an uncontrollable rate of growth. The capabilities cells acquire during the multistep progression towards malignancy have been described as the hallmarks of cancer (Hanahan and Weinberg 2011). Moreover, the role of inflammation in cancer has been widely studied, possibly contributing to up to 25% of known cancer-predisposing factors (Hussain and Harris 2007). Immune cells have long been found to infiltrate tumor tissues such that today we understand that practically all solid tumors contain immune cells. Active inflammation contributes positively to several hallmarks, such as promoting invasion and metastasis by activating the extracellular matrix, degrading enzymes, and sustaining the
proliferative signaling by supplying growth factors to the area. However, the role of inflammation in cancer is not one-sided, since an increased risk of cancer has been observed both among immunosuppressed patients and among patients with autoimmune disease (Vajdic and van Leeuwen 2009; Francescone et al. 2015).

In the context of malignant diseases, biomarkers or tumor markers form a category of molecular compounds that can be measured objectively (Nagpal et al. 2016). Such biomarkers can be expressed in the tumor tissue itself and/or they circulate in the bloodstream. Currently, tumor markers play an important role in the diagnosis of and follow-up in various cancers, including calcitonin in medullary thyroid cancer and prostate-specific antigen in prostate cancer (Duffy et al. 2014). Tumor markers can serve both prognostic and diagnostic purposes and are exceptionally useful in the treatment and diagnosis of diseases without clear clinical symptoms. In addition, they can also assist in detecting recurrence during follow-up. They may also serve as direct target molecules for specific therapies, and changes in the tumor marker levels can provide feedback regarding treatment responses.

As such, this thesis aims to evaluate the significance of a set of novel biomarkers in gastric cancer. The original studies addressed tumor tissue expressions of matrix metalloproteinase 14 (MMP14), prospero homeobox protein 1 (PROX1), and six distinct toll-like receptors (TLRs): TLR1, TLR2, TLR4, TLR5, TLR7, and TLR9. Furthermore, preoperative serum levels of MMP14, tumor-associated trypsin-2 (TAT-2), tumor-associated trypsin inhibitor (TATI), and plasma C-reactive protein (CRP) were studied in patients with gastric cancer. We studied the associations between biomarker levels, survival data, and patient clinicopathological qualities, including patient characteristics and tumor-derived factors, such as the Laurén classification, the tumor invasion depth, and the presence of metastasis.
6 REVIEW OF THE LITERATURE

6.1 Epidemiology and incidence

In the 1970s, gastric cancer was the most common type of cancer globally. Today, gastric cancer stands as the sixth most common cancer with 1 030 000 (5.7% of all cancers) new cases occurring annually across the globe (Bray et al. 2018; Etemadi et al. 2020). Gastric cancer is surpassed in incidence by skin, colon, prostate, breast, and lung cancers; however, it is the second most common cause of cancer-related mortality. Furthermore, an estimated 780 000 gastric cancer-related deaths occur annually. Only lung cancer accounts for more deaths than gastric cancer, although its incidence is more than double that of gastric cancer.

Gastric cancer associates with an older age, typically diagnosed at between 60 and 80 years of age (Ang and Fock 2014). It rarely affects patients under 40 years of age and very rarely occurs in patients under 30.

The incidence and mortality of gastric cancer are higher among men than among women and differ by geographic areas. Gastric cancer is particularly common in East Asia and Eastern Europe (Bray et al. 2018). The highest incidence occurs in the Republic of Korea, reaching nearly 60/100 000 population annually among men. These high incidence rates in East Asia and Eastern Europe may be partly explained by traditional food preservation methods, a higher H. pylori prevalence, as well by a higher alcohol consumption (Torre et al. 2015). Correspondingly, mortality rates remain high in those areas. Interestingly, incidence and mortality rates are low in Northern Europe and North America.

The incidence of cancer of the gastric cardia continues to increase, particularly in countries with a higher socioeconomic level, contrary to the decreasing incidence of noncardia tumors (Bray et al. 2018). Cancer of the cardia appears to associate with obesity and gastroesophageal reflux disease.

In Finland, gastric cancer incidence and mortality have steadily decreased in recent decades (Figures 1 and 2). In 2017, 623 new gastric cancer cases were recorded with an age-standardized incidence of 5.6/100 000 among men and 3.8/100 000 among women (The Finnish Cancer Registry 2017). In 1987, 1171 new cases were recorded, whereas 60 years earlier in 1957, 1930 cases were recorded. In 2017, the
age-standardized mortality was 4.1/100 000 among men and 2.3/100 000 among women, with 5-year survival standing at 21% and 26%, respectively.

Figure 1. The age-standardized incidence among A) men and B) women for gastric, colon, breast, lung, and prostate cancers in Finland. Adapted from The Finnish Cancer Registry (2017).
Figure 2. The age-standardized mortality among A) men and B) women for gastric, colon, breast, lung, and prostate cancers in Finland. Adapted from The Finnish Cancer Registry (2017).
6.2 Etiology and risk factors

6.2.1 Helicobacter pylori

The discovery of Helicobacter pylori (H. pylori) by Australian scientists Barry Marshall and Robin Warren in 1982, identifying its role in gastric ulcer and chronic gastritis, represented a milestone (Warren and Marshall 1983; Marshall and Warren 1984). Marshall and Warren subsequently received the 2005 Nobel Prize in Physiology or Medicine as recognition for their finding. H. pylori infection was established as the most important risk factor of developing gastric cancer, particularly in the gastric antrum (Polk and Peek 2010).

H. pylori infection is common, especially in developing countries. Estimates suggest that up to half of the global population carries the pathogen (Linz et al. 2007). Infection is often acquired in childhood, and without antimicrobial treatment can persist for a lifetime (Wroblewski et al. 2010). H. pylori infection predisposes an individual to atrophic gastritis, gastric cancer, and gastric lymphoma. In populations with 100% H. pylori prevalence, the risk of gastric cancer is sixfold that among populations without H. pylori colonization of the stomach (Helicobacter and Cancer Collaborative Group 2001). Moreover, animal studies have shown that H. pylori infection of the gastric mucosa increase the incidence of adenocarcinoma, while eradication of H. pylori through antimicrobial medication lowers the risk of developing cancer (Tsukamoto et al. 2013).

Recently, a large retrospective cohort study among 371,813 individuals from the United States with a history of H. pylori infection showed that treatment reduces the risk of developing gastric cancer, although only if eradication succeeds (Kumar et al. 2020). In that study, the primary outcome was diagnosis of distal gastric adenocarcinoma 30 days or more following H. pylori detection. Men (92.3% of the study cohort) exhibited a heightened risk of developing cancer compared to women. Additional factors associated with gastric cancer accompanying H. pylori infection included an older age, ethnically being African American, Asian, Hispanic, or Latino, and tobacco smoking.

Variables affecting the outcome of H. pylori infection include H. pylori virulence factors. The most important H. pylori virulence factors include cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA). H. pylori induces the formation of gastric cancer by promoting defective autophagy of the gastric epithelial cells through CagA and VacA functioning (Eslami et al. 2019). Defective autophagy leads
to the accumulation of cytotoxic debris within the epithelial cells, thereby predisposing individuals to genomic mutations. VacA and CagA may also disrupt epithelial junctions. Previously, CagA-positive *H. Pylori* strains appeared to induce a 5.8-fold risk for developing cancer compared to uninfected individuals, whereas CagA-negative strains induced a 2.2-fold risk for developing cancer (Parsonnet et al. 1997). Variations in *H. pylori* virulence factors also appear to contribute to explaining the high *H. pylori* prevalence accompanied by a low gastric cancer incidence in certain regions, such as sub-Saharan Africa (Ang and Fock 2014).

Chronic *H. pylori* infection contributes to gastric carcinogenesis both via the virulence factors and by inducing a local inflammatory reaction (Qadri et al. 2014). In addition, *H. pylori* infection activates toll-like receptors (TLRs), resulting in an activated Cyclooxygenase-2 (COX-2)/Prostaglandin E2 (PGE2) axis, possibly also inducing epigenetic alterations, particularly deoxyribonucleic acid (DNA) methylation (Matususaka et al. 2014; Echizen et al. 2016). The interplay between *H. pylori*-induced inflammation and gastric cancer stem cell recruitment seems also suitably documented (Ferrand et al. 2011).

### 6.2.2 Epstein–Barr virus (EBV)

Another microbe associated with gastric cancer is the well-known cause of mononucleosis, the Epstein–Barr virus (EBV). EBV infection is found in 5% to 17% of gastric cancer tissues, while absent or quite rare in adjacent healthy tissue (Chen et al. 2015). EBV positivity also more commonly occurs among patients with cancer of the gastric cardia, who underwent previous gastric surgery, with a younger age, and who are male (Lee et al. 2009). In addition, EBV-positive gastric cancers appear to carry a better prognosis. This may be explained by a potential immunological background; cytotoxic CD8 lymphocytes may be more efficient in eradicating EBV-positive malignant cells than EBV-negative cells (Saiki et al. 1996).

### 6.2.3 Lifestyle, dietary, and environmental factors

Alongside *H. pylori* infection, several lifestyle factors predispose individuals to gastric cancer (Table 1). Tobacco smoking is well-known to increase the risk of several malignant diseases, including gastric cancer (Gandini et al. 2008). Tobacco-derived carcinogens locally affect the stomach, once dissolved and swallowed with saliva and absorbed via the systemic circulation. Tobacco smoking predisposes individuals to gastric cancer in a dose-dependent manner and the risk of developing
gastric cancer is elevated among tobacco smokers regardless of race or sex (Sauvaget et al. 2005; Nomura et al. 2012). Multiple studies have reported a intestinal-type histology as more common among patients with gastric cancer with a history of smoking, although further studies are needed to make definitive conclusions, since this association is not found in some cohorts (Sasazuki et al. 2002; Nomura et al. 2012). In addition, alcohol consumption has been well reported as possibly amplifying the carcinogenic effects of tobacco smoking (Inoue et al. 1994; Sjödahl et al. 2007).

**Table 1. Factors associated with an increased risk of gastric cancer.**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Risk Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. pylori infection</td>
<td>Tobacco smoking</td>
</tr>
<tr>
<td></td>
<td>Alcohol consumption</td>
</tr>
<tr>
<td></td>
<td>Consumption of salted, pickled, and smoked foods</td>
</tr>
<tr>
<td></td>
<td>Obesity</td>
</tr>
</tbody>
</table>

The relationship between alcohol consumption and gastric cancer has been extensively studied, such that strong evidence exists showing that alcohol consumption predisposes an individual to gastric cancer. For instance, ethanol metabolizes to acetaldehyde, thus becoming capable of disturbing the mucosal balance in the epithelial lining of the stomach, causing an increased oxidative stress and exposure to malignant transformations (Kwiecien et al. 2002). Some studies have found that high alcohol consumption associates with gastric cancer despite the anatomical subsite; other studies merely found an association with cancers of the cardia or the upper third of the stomach (Tramacere et al. 2012; Massarrat and Stolte 2014). A recent meta-analysis concluded that even minor alcohol consumption increases the risk for gastric cancer despite the anatomical subsite (Ma et al. 2017). High alcohol consumption predisposes individuals to esophageal cancer. Cancers of the gastroesophageal junction (GEJ) share features with both esophageal and gastric cancers. This may partly explain why in some studies the association between alcohol consumption and gastric cancer is only seen in cancers of the cardia.

Consuming foods preserved by smoking, pickling, and salting in particular predisposes individuals to gastric cancer (Sitarz et al. 2018). In a large meta-analysis of more than 2 million patients, the risk of developing gastric cancer was twofold greater among individuals with a high dietary salt intake compared to those with a low salt intake (Ge et al. 2012). Furthermore, fresh fruit and vegetables carry antioxidative qualities, and their consumption associates with a lower risk of developing gastric cancer (Riboli and Norat 2003; Larsson et al. 2006; Guggenheim and Shah 2013).
In 2015, 604 million adults and 108 million children were obese, and obesity continues increasing globally (Afshin et al. 2017). Moreover, obesity associates with an increased risk for and mortality associated with multiple cancers, including gastric cancer, particularly of the cardia (Calle et al. 2003). Calle et al. argued that 14% of all cancer-related mortality in men and 20% in women may be attributable to obesity.

6.2.4 Hereditary gastric cancer

A family history of gastric cancer in first- and second-degree relatives is a strong risk factor, elevating the risk of acquiring gastric cancer threefold compared to individuals without a family history of disease (Choi and Kim 2016). Most gastric cancers are sporadic, although we see a family history of disease in up to 10% of gastric cancers (Yaghoobi et al. 2010; Kluijt et al. 2012). Familial clustering or familial gastric cancer often results from shared environmental and lifestyle factors. Hereditary syndromes have been identified as a cause or a strong predisposing factor in only approximately 1% to 3% of gastric cancers (Colvin et al. 2015; Setia et al. 2015; Table 2). Gastric cancer predisposing hereditary syndromes are numerous and can be further divided into polyposis-associated and nonpolyposis syndromes.

Nonpolyposis syndromes include hereditary diffuse gastric cancer (HDGC), familial intestinal gastric cancer, Lynch syndrome, and Li–Fraumeni syndrome. HDGC strongly increases the risk of diffuse-type gastric cancer, and results from an autosomal dominantly inherited mutation of the epithelial cadherin (E-cadherin) coding gene (CDH1) (Kaurah et al. 2007). E-cadherin is a cell surface protein that plays a crucial role in cell–cell adhesions; when lacking, it highly predisposes an individual to diffuse-type gastric carcinoma and to lobular breast cancer. HDGC syndrome does not predispose an individual to intestinal-type gastric carcinoma. Currently, a prophylactic complete gastrectomy is recommended for HDGC patients 20- to 30-years old, since this inherited form of diffuse gastric cancer typically develops at a young age (Polom et al. 2018a).

Polyposis-associated syndromes include familial adenomatous polyposis (FAP), juvenile polyposis syndrome, gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS), and Peutz–Jeghers syndromes.
Table 2. Gastric cancer predisposing hereditary syndromes (Colvin et al. 2015; Setia et al. 2015).

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Gene</th>
<th>Gastric cancer risk</th>
<th>Other associated cancers</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDGC</td>
<td>CDH1</td>
<td>Up to 70%</td>
<td>Lobular breast carcinoma, colorectal, prostate</td>
</tr>
<tr>
<td>Peutz–Jeghers syndrome</td>
<td>STK11</td>
<td>Up to 30%</td>
<td>Breast, pancreatic, lung, colorectal, ovarian, testicular</td>
</tr>
<tr>
<td>Juvenile polyposis syndrome</td>
<td>SMAD4</td>
<td>Up to 21%</td>
<td>Colorectal, duodenal</td>
</tr>
<tr>
<td>Lynch syndrome</td>
<td>MLH1, MSH2, MSH6, PMS2</td>
<td>Up to 13%</td>
<td>Colorectal, endometrial, ovarian, other</td>
</tr>
<tr>
<td>Li–Fraumeni syndrome</td>
<td>TP53</td>
<td>Up to 5%</td>
<td>Breast, colorectal, lung, thyroid, other</td>
</tr>
<tr>
<td>FAP</td>
<td>APC</td>
<td>Less than 1%</td>
<td>Colorectal, thyroid</td>
</tr>
<tr>
<td>GAPPS</td>
<td>APC promoter 1B</td>
<td>Unclear</td>
<td>Unclear</td>
</tr>
<tr>
<td>Familial intestinal gastric cancer</td>
<td>Unknown</td>
<td>Unclear</td>
<td>Unclear</td>
</tr>
</tbody>
</table>

Abbreviations: HDGC, hereditary diffuse gastric cancer; FAP, familial adenomatous polyposis; GAPPS, gastric adenocarcinoma and proximal polyposis of the stomach

6.3 Pathogenesis

6.3.1 Cellular basis of carcinogenesis

Today, the somatic mutation theory (SMT) stands as the prevailing explanation for carcinogenesis. According to SMT, cancer is fundamentally a problem of uncontrollable cell proliferation (Vicente-Dueñas et al. 2013). Once a cell acquires sufficient oncogenic mutations to break through growth-controlling mechanisms, it begins to proliferate uncontrollably. Uncontrollable proliferation leads to an excess of cells and eventually to impaired tissue organization and function. In addition to a burden of oncogenic mutations, an absence of tumor suppressors is required for carcinogenesis to take place. Moreover, high levels of reactive oxygen species promote the production of free radicals, which may subsequently cause DNA damage (Saha et al. 2017). Most human tumors express a high level of heterogeneity with various inherited and sporadic mutations (Tabassum and Polyak 2015). Thus, as swift clonal expansion continues, mutations promoting advantageous cell phenotypes generalize resulting in a tumor comprised of highly malignant cells (Chaffer and Weinberg 2015).
Malignant tumors appear to originate from cancer stem cells, an idea first proposed as the cells-of-origin more than 40 years ago (Hamburger and Salmon 1977b; Hamburger and Salmon 1977a). These represent a small subpopulation of malignant cells, originating either from healthy stem or differentiated cells capable of sustainable proliferation. The epithelial–mesenchymal transition (EMT) is a process in which epithelial cells undergo transformation into mesenchymal cells with stem cell properties (Kalluri and Weinberg 2009). The epithelial cells lose their cell–cell adhesions and polarity, and become capable of migrating through the basement layer. EMT is a common process in embryonic development, but also occurs in cancer. However, in cancer, EMT is not always completed and may result in cells expressing mixed epithelial–mesenchymal phenotypes (Wang and Unternaehrer 2019). Nevertheless, today EMT is regarded as an important source of cancer stem cells. Several factors contribute to the induction of EMT, particularly local growth factors and cytokines, hypoxia, and immune responses (Roche 2018).

6.3.2 Histopathology of gastric cancer

The majority of malignant gastric tumors are carcinomas, but also include lymphomas, mesenchymal tumors, and rare neuroendocrine tumors. Gastric carcinomas include adenocarcinomas and the rarer squamous cell carcinomas, adenosquamous carcinomas, and undifferentiated carcinomas (WHO Classification of Tumors 2019). Approximately 90% of gastric malignancies consist of adenocarcinomas. Intestinal-type adenocarcinoma is thought to develop from a sequence of precancerous lesions, also known as the Correa pathway (Correa and Shiao 1994; Correa and Piazuelo 2012; Figure 3). The Correa pathway begins with active chronic inflammation of the gastric mucosa, followed by atrophic gastritis, intestinal metaplasia, dysplasia, and, finally, cancer. Inflammation of the gastric mucosa may present as superficial or atrophic gastritis. Superficial gastritis does not present as a multifocal loss of glandular structures, on the contrary to atrophic gastritis. Superficial gastritis can progress to atrophic gastritis, predisposing an individual to cancer.
Figure 3. A schematic representation of the Correa pathway. *H. pylori* infection, reactive oxygen and nitrogen species, and lifestyle and environmental factors contribute to the sequential chain of events.

Intestinal metaplasia of the gastric mucosa presents as a reconfiguration of glandular structures (Kinoshita et al. 2017), and falls within the complete and incomplete types. Complete intestinal metaplasia is characterized by intestinal glands with brush-bordered enterocytes, goblet cells, and Paneth cells, and appears to carry a lower risk of progressing to dysplasia and cancer (González et al. 2016). The incomplete type presents with an irregular mixture of mature goblet cells and immature mucous cells. Whether incomplete intestinal metaplasia is a consequence of complete intestinal metaplasia or if it occurs independently remains uncertain. Gastric dysplasia is divided into the low- and high-grade types. High-grade dysplasia is the final step before pT1 carcinoma, characterized by the invasion of the lamina propria.

Patients with hyperplastic and adenomatous gastric polyps are at an increased risk of developing intestinal gastric adenocarcinoma (Goddard et al. 2010). They are not dysplastic themselves but can progress. Gastric adenomas are histologically classified into tubular, tubulovillous, and villous adenomas. The risk of progression to cancer is high in villous and tubulovillous adenomas (Stolte 1995; Schmitz and Stolte 1997).
6.4 Classifications

6.4.1 Anatomical classification

Tumor location, an important factor in gastric cancer, substantially affects the surgical treatment available. The stomach is anatomically divided into several subsites — the cardia, fundus, corpus, antrum, and pylorus, as well as the greater and the lesser curvatures. The GEJ separates the cardia from the esophagus. Cancers with a midpoint more than 5 cm distally from the GEJ and cancers with a midpoint less than 5 cm from the GEJ, but which do not extend into the GEJ are classified as gastric cancers (Brierley et al. 2017). Furthermore, gastric cancers are classified as proximal, middle, and distal third tumors. GEJ tumors are classified according to the Siewert classification (Figure 4). Tumors with a midpoint 1 to 5 cm proximal to the GEJ are classified as type I. Tumors with a midpoint ranging from 1-cm proximal to 2-cm distal to the GEJ are classified as type II. Tumor with a midpoint 2 to 5 cm distal to the GEJ are classified as type III.

![Diagram of stomach showing anatomical classification](image)

**Figure 4.** Representation of the Siewert classification of gastroesophageal tumors. Reproduced with permission from Springer Nature (Ajani et al. 2017).
6.4.2 The Laurén and WHO classifications

The Finnish pathologist Pekka Laurén established a histological classification of gastric cancer in 1965, which has since been in active clinical use (Lauren 1965). The Laurén classification divides gastric cancers into intestinal-type, diffuse-type, and mixed-type tumors. Intestinal-type carcinomas often develop via the Correa pathway with a contributing H. pylori infection (Tahara 2004). Intestinal-type carcinomas are more organized, form glandular structures, and are commonly found in the proximal stomach, near the GEJ, and in the cardia (Hansen et al. 2007). Diffuse-type carcinomas consist of noncohesive malignant cells lacking gland formation. Diffuse gastric carcinoma is characterized by a mutated CDH1 gene, resulting in impaired E-cadherin, and, thus, impaired cell–cell adhesions (Carneiro et al. 2004). Signet ring cells are distinct diffuse-type gastric cancer cells with large intracellular mucin vacuoles that push the nucleus and cytoplasm to the cell periphery (Pernot et al. 2015). Signet ring cells associate with a poor prognosis.

The World Health Organization (WHO) classification divides gastric adenocarcinomas into papillary, tubular, mucinous, poorly cohesive (including the signet ring cell carcinomas), and uncommon histological variants (WHO Classification of Tumors, 2019). The WHO classification is based on the dominant histological morphology; however, several traits can often be seen.

6.5 Diagnosis

6.5.1 Symptoms

The early diagnosis of gastric cancer is challenging, whereby the majority of early gastric cancers remain undiagnosed. Upon diagnosis, treatment with a curative intent is possible for less than half of all patients. During the early stages, gastric cancer accompanies no symptoms and can only be diagnosed through endoscopic screening with sufficient biopsies. Symptoms appear late, are nonspecific, and overlap with symptoms of benign disease. Alarming features include dysphagia, vomiting, early satiety, a family history of gastric cancer, and unintended weight loss (Talley et al. 2005). In patients with such symptoms, eradicating H. pylori after a false-negative upper gastrointestinal endoscopy or no endoscopy at all may delay detection of gastric cancer (Kokkola et al. 2008). Ulcerated tumors may result in either acute or chronic gastrointestinal bleeding. At times, iron deficiency anemia may represent the only clinical sign of a chronically bleeding tumor. Severe clinical manifestations
including palpable masses of the abdomen, pelvis, or liver, jaundice, extensive ascites, or typical prominent lymph nodes do not present until an advanced stage of disease (Catalano et al. 2009; Thrumurthy et al. 2013). The formation of metastases radically worsens the outcome: an early diagnosis is essential to achieving a favorable prognosis.

Tumors of the proximal stomach present with dysphagia and vomiting more often than distal tumors. Textbook examples, including a prominent left-supraclavicular lymph node: the Virchow’s nodule, or a prominent periumbilical node: Sister Mary Joseph’s nodule, do not present until metastasis.

6.5.2 Upper gastrointestinal endoscopy

An esophagogastroduodenoscopy (EGD) with biopsies from suspicious lesions and adjacent tissues plays a crucial role in diagnosing gastric cancer (Smyth et al. 2016). EGD allows for the visualization of the tumor size, location, and appearance through imaging of the findings. EGD findings are central to planning surgical treatment, presuming that treatment with a curative intent is otherwise indicated. An urgent EGD is needed among patients with alarming symptoms, serving as the only means of decreasing the number of inoperable patients at the time of diagnosis.

6.5.3 Preoperative staging

Perioperative staging is vital in assessing whether a patient is suitable for radical surgery. Computed tomography serves to determine the preoperative disease stage. However, it cannot accurately identify small lymph node metastases and local dissemination (Allum et al. 2011; Thrumurthy et al. 2013). In order to produce a high-quality image of the gastric wall, the ventricle must be filled with either air or liquid upon imaging. Endoscopic ultrasound is helpful in regional staging, whereas a positron emission tomography (PET) registry may aid in detecting distant metastasis (Mocellin et al. 2011; Marcus and Subramaniam 2017). PET imaging may also prove promising in evaluating the response to neoadjuvant treatment in gastric cancer, esophageal cancer, and cancers of the GEJ (Lordick et al. 2012). However, conflicting findings exist. For instance, Vihervaara et al. (2019) found no association between a favorable histopathological response to preoperative chemotherapy and PET imaging among 54 patients with adenocarcinoma of the distal esophagus, GEJ, or stomach. Preoperative staging via laparoscopy represents an additional diagnostic tool for detecting peritoneal metastases if other investigations indicate treatment with
a curative intent, although clinical doubt exists regarding metastatic disease (Thrumurthy et al. 2013). Staging laparoscopy has been reported altering treatment in over 20% of patients with gastric cancer undergoing the procedure (Convie et al. 2015).

6.6 Treatment

Curative treatment of gastric cancer always requires surgery. The recommended treatment should be determined by a multidisciplinary team consisting of a surgeon, an oncologist, a radiologist, and a pathologist taking into account all of the essential parameters. These parameters consist of the stage of disease, patient age, comorbidities, and physical fitness.

6.6.1 Surgery

6.6.1.1 Surgery with a curative intent

Surgery with a curative intent requires an R0 resection, characterized by the removal of all cancerous tissue with both macroscopically and microscopically margins clear of malignant cells (Hermanek and Wittekind 1994). An R1 resection is characterized by a clear macroscopic view, but with residual malignant cells at the resection margin in the histopathological examination. In an R2 resection, removal of the entire tumor is impossible, leaving a macroscopic residual (metastasis or macroscopic marginal residual).

Generally, stage IA lesions can be treated using endoscopic resection alone, while more advanced operable tumors may require total or subtotal gastrectomy accompanied by adjuvant therapy (Smyth et al. 2016; Orditura et al. 2014; Figure 5). In the literature, a subtotal gastrectomy is also referred to as a distal gastrectomy. Distal tumors can be treated through a subtotal gastrectomy if the proximal margin for intestinal tumors measures at least 5 cm macroscopically and at least an 8-cm proximal margin for diffuse tumors is achieved between the tumor and the GEJ. In other cases, total gastrectomy is required. Comparing patients who underwent subtotal versus total gastrectomies, no differences in the survival rates are observed when an R0 resection is verified and a subtotal gastrectomy is performed with sufficient margins (Smyth et al. 2016). Yet, for suitable patients, a subtotal gastrectomy remains the preferred surgery since it associates with a better nutritional status and a better overall quality of life.
Figure 5. A) Total and B) subtotal gastrectomies with reconstruction techniques. Reproduced with permission from Duodecim (Leppäniemi et al. 2018). Figure drawn by Tiina Ripatti-Toledo.

Gastric cancer can be treated either laparoscopically or via open surgery. Laparoscopy was first used in the treatment of gastric cancer in Asia, whereby only small and distal tumors were treated laparoscopically. Today, the laparoscopic treatment of more advanced gastric cancers has also become common. In general, short-term results for both total and subtotal gastrectomies favor the laparoscopic technique (Liao et al. 2020; Korean Laparoendoscopic Gastrointestinal Surgery Study (KLASS) Group 2016; 2019a). Laparoscopic surgery associates with a
quicker return of the gastrointestinal function, less postoperative pain, fewer wound complications, and less intra- and postoperative bleeding. Recent studies and meta-analyses also demonstrate that the long-term results of laparoscopy are comparable to open surgery, such that laparoscopic surgery remains an oncologically safe alternative (KLASS Group 2012; 2019b; Beyer et al. 2019; Li et al. 2019a). Currently, several ongoing clinical trials are further evaluating the long-term results of the two procedures, particularly among those with locally advanced disease.

A curative resection involves the removal of all cancer cells harboring lymph nodes. The degree of lymph node dissection remains a key feature of gastric cancer surgery (Smyth et al. 2016), and is classified from D0 to D4. In D0, no nodes are removed (mostly considered in palliative surgery). In D1, the perigastric lymph nodes are removed, whereas in D2, the nodes are also removed along the neighboring large arteries, including the celiac artery, the splenic artery, the left gastric artery, and the common hepatic artery (Figure 6 and Table 3). D3 and D4 lymphadenectomies are not routinely performed and appear not to offer a survival benefit (Sasako et al. 2008). In D3, additional nodes are removed along the superior mesenteric artery and vein, the portal vein, and from the retropancreatic region. In D4, the para-aortic nodes and nodes around the porta hepatitis and vena cava are also removed.

The benefit of D1 versus D2 lymphadenectomies stems from an actively researched topic in recent decades. Initial results from a Western randomized controlled trial addressing D1 versus D2 lymphadenectomies in gastric cancer indicated a higher mortality following a D2 lymphadenectomy (Bonenkamp et al. 1995). However, among the D2 group, more splenectomies and pancreatectomies were performed, thereby increasing postoperative mortality (Cuschieri et al. 1996). In that study, after a 15-year follow-up, no differences were identified in the overall survival, and gastric cancer–specific mortality was actually significantly lower among the D2 group (Songun et al. 2010). This indicates that D2 lymphadenectomy serves as an oncologically beneficial alternative, representing the preferred treatment option if postoperative mortality can be avoided. Today, D2 lymphadenectomy is the gold standard for surgery performed in high-quality centers (Schmidt and Yoon 2013; Garg et al. 2016).
Figure 6. Lymph node areas of the stomach. D1 and D2 lymphadenectomies include removal of lymph node areas 1–12a. Lymph node areas 13–16 (D2+/D4) are not routinely removed. Reproduced with permission from Duodecim (Leppäniemi et al. 2018). Figure drawn by Tiina Ripatti–Toledo.

Table 3. Lymph node areas removed in D1 and D2 lymphadenectomies. Adapted from Duodecim Kirurgia, third edition (Leppäniemi et al. 2018).

<table>
<thead>
<tr>
<th>Total gastrectomy</th>
<th>D1</th>
<th>Lymph node areas 1–7</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1+</td>
<td>D1</td>
<td>and lymph node areas 8a, 9, 11p</td>
</tr>
<tr>
<td>D2</td>
<td>D1</td>
<td>and lymph node areas 8a, 9, 10, 11p, 11d, 12a</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Subtotal gastrectomy</th>
<th>D1</th>
<th>Lymph node areas 1, 3, 4sb, 4d, 5, 6, 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1+</td>
<td>D1</td>
<td>and lymph node areas 8a and 9</td>
</tr>
<tr>
<td>D2</td>
<td>D1</td>
<td>and lymph node areas 8a, 9, 11p, 12a</td>
</tr>
</tbody>
</table>

Avoiding postoperative mortality is central to the treatment of gastric cancer. Gastric cancer surgery and the postoperative care remains challenging, and several studies have concluded that postoperative mortality is lower and survival better in experienced centers with a high volume of patients (Dikken et al. 2013). Thus, the treatment of gastric cancer should be centralized, particularly in countries with a lower incidence. In Finland, gastric cancer treatment is centralized to five university hospitals.

Robotic laparoscopy has been widely adopted in gynecology and urology, but not in upper gastrointestinal surgery given the lack of evidence of its superiority compared to manual laparoscopy. Costs remain higher and surgery duration is longer, although the high precision has been speculated to improve the meticulousness of lymph node

**6.6.1.2 Endoscopic treatment**

Small (<20 mm diameter) stage IA gastric cancers can be treated through an endoscopic technique, endoscopic mucosal resection, or endoscopic submucosal dissection. Endoscopic mucosal resection can be considered for very small pT1a tumors (<10–15 mm diameter), although endoscopic submucosal dissection is the recommended procedure in most cases (Pimentel–Nunes et al. 2015). Patients suitable for endoscopic submucosal dissection enjoy an exceptionally good prognosis, with 5-year survival typically reaching over 95%, although patients must be carefully selected to verify the absence of lymph node metastasis (Espinell et al. 2015). Additionally, meticulous histopathological examination of the surgical specimen must be carried out to verify an R0 resection.

**6.6.1.3 Palliative surgery**

Inoperable or metastatic gastric cancers require palliative care. Palliative care aims to improve the quality of any remaining life. Surgical treatment is not routinely recommended, although palliative surgery may be required in some cases in order to relieve symptoms (Smyth et al. 2016). The most common forms of palliative surgery in gastric cancer include the application of self-expanding metallic stents (SEMSes) and the construction of bypasses to treat obstruction (Seshadri and Glehen 2016).

**6.6.2 Oncological treatment**

An R0 resection should represent a curative treatment with a low probability of recurrence and a favorable prognosis. Yet, the overall 5-year survival of surgically treated patients with gastric cancer stands at less than 50%. This likely stems from the presence of micrometastases within other organs or the peritoneal surfaces, resulting in recurrence (Biondi et al. 2010). Thus, oncological treatment in addition to surgery is crucial to treating gastric cancer.

According to current European guidelines, perioperative (pre- and postoperative) chemotherapy with a platinum fluoropyrimidine–based drug combination is recommended for patients with an operable stage IB or more advanced disease (Smyth et al. 2016). The perioperative approach appears most beneficial, as long as
the patient’s fitness allows for the administration of postoperative chemotherapy according to the original treatment plan. Evidence from randomized trials supports this plan. For instance, the MAGIC trial showed that a perioperative chemotherapy regimen of three cycles pre- and three postoperatively significantly improved prognosis among patients with gastric cancer with stage II or III disease compared to surgery alone (Cunningham et al. 2006). In the MAGIC trial, the resected tumors were significantly smaller and less advanced among patients who received perioperative chemotherapy. Ychou et al. (2011) in a similar study also found that the risk of recurrence was lower among patients who received perioperative chemotherapy. Thus, perioperative chemotherapy appears beneficial in enhancing operability by reducing the tumor size. The response to preoperative oncological treatment may be evaluated through PET imaging (Lordick 2012).

Postoperative chemotherapy or chemoradiotherapy is recommended in stage IB or more advanced gastric cancers that have been operated on without administering preoperative chemotherapy (Smyth et al 2016; Verheij et al. 2016). Postoperative chemotherapy appears to increase the 5-year survival among operable patients with gastric cancer by 6% in comparison to patients treated by surgery alone (Paoletti et al. 2010). Chemoradiotherapy may prove beneficial only if the lymphadenectomy was insufficient (Smyth et al. 2016). Thus, chemoradiotherapy does not appear to reduce the risk of recurrence if a D2 lymphadenectomy was performed (Dikken et al. 2010). However, this remains the subject of active research since some studies also showed that additional postoperative radiotherapy may be beneficial even after a D2 lymph node dissection among specific subgroups of patients (Park et al. 2015; Zhu et al. 2012). Moreover, evidence indicates that adjuvant postoperative chemoradiotherapy improves prognosis following an R1 resection (Stiekema et al. 2014).

Chemotherapy remains the first-line treatment for patients with inoperable gastric cancer. Trastuzumab improves prognosis and is recommended for human epidermal growth factor receptor 2 (HER2) positive inoperable patients along with chemotherapy (Smyth et al. 2016). Yet, a subpopulation of patients responded to treatment exceptionally well, possibly proving suitable for subsequent surgery. The second-line therapy for advanced gastric cancer includes treatment with taxanes, the vascular endothelial growth factor 2 (VEGF-2) antibody ramucirumab, irinotecan, and radiotherapy. Finally, participation in ongoing clinical trials investigating novel anticancer pharmaceuticals is a noteworthy option for gastric cancer patients with late-stage disease (Lordick et al. 2017).
6.7 Prognostic factors

6.7.1 Staging

The most important factor determining treatment and prognosis is the Tumor, Node, Metastasis (TNM) classification (Table 4). The eighth edition is currently in use, although many retrospective studies include patients classified based on the seventh edition (Sobin et al. 2009; Brierley et al. 2017; Table 5). In this classification, disease stage is summarized from the individual T, N, and M categories. The T category depicts the tumor size and invasion depth, N reflects the regional lymph nodes, and M refers to distant metastasis. The prefix ‘c’ shows that the stage was determined before treatment, such as via radiological imaging, and is implied in the absence of ‘p’. The prefix ‘p’ indicates that the stage was determined by the histopathological examination of a tissue sample. The prefix ‘y’ is used when the stage is assessed following the administration of neoadjuvant treatment. Figure 7 summarizes the five-year survival of patients with gastric cancer classified using the seventh and eighth editions of the TNM classification (Kim et al. 2017).

Table 4. TNM classification of gastric cancer. Adapted from the TNM Classification of Malignant Tumors, eighth edition (Brierley et al. 2017). Dissection of a minimum of 15 lymph nodes is recommended for reliable staging.

<table>
<thead>
<tr>
<th>Primary Tumor (T)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>Primary tumor cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumor</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in situ: no invasion of the lamina propria, high-grade dysplasia</td>
</tr>
<tr>
<td>T1</td>
<td>Invasion of lamina propria: muscularis mucosae (T1a) or submucosa (T1b)</td>
</tr>
<tr>
<td>T2</td>
<td>Invasion of muscularis propria</td>
</tr>
<tr>
<td>T3</td>
<td>Invasion of subserosa</td>
</tr>
<tr>
<td>T4</td>
<td>Invasion of serosa (T4a) or adjacent structures (T4b)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Regional Lymph Nodes (N)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NX</td>
<td>Regional lymph nodes cannot be assessed</td>
</tr>
<tr>
<td>N0</td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td>N1</td>
<td>Metastasis in 1–2 regional lymph nodes</td>
</tr>
<tr>
<td>N2</td>
<td>Metastasis in 3–6 regional lymph nodes</td>
</tr>
<tr>
<td>N3</td>
<td>Metastasis in more than 7 regional lymph nodes: 7 to 15 (N3a), over 15 (N3b)</td>
</tr>
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<thead>
<tr>
<th>Distant Metastasis (M)</th>
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<tbody>
<tr>
<td>MX</td>
<td>Distant metastasis cannot be assessed</td>
</tr>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
</tbody>
</table>
Table 5. Gastric cancer TNM staging. Adapted from the American Joint Committee on Cancer (AJCC) Cancer Staging Manual, seventh and eighth editions (Edge et al. 2010; Amin et al. 2017).

<table>
<thead>
<tr>
<th>TNM staging, seventh edition</th>
<th>TNM staging, eighth edition</th>
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<tbody>
<tr>
<td>Stage</td>
<td>T</td>
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<td>0</td>
<td>Tis</td>
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<tr>
<td>IA</td>
<td>1</td>
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<td>IB</td>
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<td>IIA</td>
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<td>IV</td>
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6.7.2 Biomarkers

Carbohydrate antigen (CA) 19-9 (CA19-9), CA 72-4 (CA72-4), and the carcinoembryonic antigen (CEA) represent widely studied biomarkers in gastric cancer. Yet, their sensitivity and specificity remain inadequate for routine clinical use in diagnostics, although they may prove useful as monitoring tools and in predicting prognosis (Liu et al. 2016; Kotzev and Draganov 2018). Equations with information on different biomarker levels combined can more accurately predict prognosis than the individual biomarker levels alone (Carpelan-Holmström et al. 2002; Liu et al. 2016).
Figure 7. Five-year overall survival of patients with gastric cancer classified using the seventh and eighth editions of the TNM classification. Reproduced with permission from the Korean Gastric Cancer Association (Kim et al. 2017).

As many as half of all patients with gastric cancer have exhibited HER2 positivity (Boku 2014; Wang et al. 2017a). A high HER2 expression predicts a poor prognosis and, thus, serves as a fitting target for therapy. Trastuzumab efficaciously antagonizes HER2 and improves prognosis among patients with gastric cancer with advanced disease. Combining trastuzumab with chemotherapy improves prognosis compared to chemotherapy alone (Bang et al. 2010). HER2 antagonists are a considerable advancement and currently the only form of targeted therapy in routine practice in gastric cancer.

6.7.3 Other prognostic factors

Prognosis is worse among patients with diffuse gastric carcinoma. This results from the lack of cohesion as small infiltrative cells readily form metastases and disease progresses quickly (Lauren 1965; Van Cutsem et al. 2016; Ansari et al. 2018).

Proximal tumors require more extensive surgery than distal tumors, although multicenter studies have shown that prognosis does not vary between the two different surgical procedures (Gouzi et al. 1989; Bozzetti et al. 1999). Still, proximal tumors associate with a more advanced stage and, hence, may feature a worse prognosis (Piso et al. 2000; Pacelli et al. 2001).
6.8 The hallmarks of cancer

The abilities cells acquire during the multistep progression from healthy to malignant were originally described as the hallmarks of cancer by Douglas Hanahan and Robert Weinberg in 2000 with revisions in 2011. These revised hallmarks of cancer include eight biological capabilities: sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, resisting apoptosis, reprogramming energy metabolism, and avoiding immune destruction (Hanahan and Weinberg 2011; Figure 8). Each capability is fundamental to understanding the mechanisms around the development and progression of malignant diseases. Genomic instability and tumor promoting inflammation represent important contributing factors. Hanahan and Weinberg suggested that the great majority if not all types of human tumors must acquire these hallmark capabilities. However, the chronological sequence and relevance of genetic alterations may differ among different tumors.

![Diagram of hallmarks of cancer with potential therapies](image)

**Figure 8.** The hallmarks of cancer with the potential therapies. Reproduced with permission from Elsevier (Hanahan and Weinberg 2011).
Several molecular mechanisms are commonly shared among multiple malignancies. The typical mechanisms of continuous growth stimulus include activated oncogenic Ras signaling, whereas an insensitivity to tumor-suppressing signals may result from the loss of function of the tumor-suppressor retinoblastoma protein. Tumors can evade apoptosis through p53 mutations. Furthermore, replicative immortality commonly results from activated telomerase. The production of excess VEGF induces angiogenesis and a dysfunctional E-cadherin protein may result in faulty cell–cell adhesions enabling tissue invasion and metastasis.

Genomic instability is a prerequisite for the occurrence of random mutations (Lengauer et al. 1998). Flaws in the genomic maintenance machinery promote a genomic instability and increase the mutation frequency. Currently, a dysfunctional p53 signaling pathway incapable of steering cells with DNA damage into DNA repair or apoptosis is found in nearly all malignant diseases. Increased apoptosis appears to increase the genomic instability via the phagocytosis of apoptotic DNA (Holmgren et al. 1999). Additionally, the loss of telomeric DNA may promote genomic instability through alterations in the entire chromosomal segments. Thus, the function of telomerase extends from enabling replicative immortality to preserving the genomic stability (Maciejowski and de Lange 2017).

Cancer cells induce angiogenesis in order to receive ample nutrients and oxygen to fuel their rapid proliferation. However, the neovascularity of malignant tumors is chaotic and most tumors are largely hypoxic within and in order to produce ample energy for growth and division under anaerobic conditions; likewise, malignant cells readjust their energy metabolism to favor glycolysis even in the presence of oxygen. This reprogrammed energy metabolism in cancer cells is referred to as aerobic glycolysis, or the Warburg effect, to honor the Nobel Laureate Otto Warburg, who first described the phenomenon in 1927 (Warburg et al. 1927).

6.9 Molecular pathways of gastric carcinogenesis

Gastric carcinogenesis is a complex process involving numerous genetic and epigenetic alterations, including single nucleotide mutations, chromosomal translocations and deletions, an abnormal nucleosome positioning, gene methylations, and histone modifications (Shi et al. 2014).
6.9.1 Intestinal- and diffuse-type gastric cancer

Distinct genetic alterations are more common among either diffuse- or intestinal-type tumors. A diffuse-type histology appears not to arise from a specific carcinogenesis sequence, although certain molecular traits can be identified. The dysfunction of E-cadherin, coded by the CDH1 gene, is a characteristic feature in diffuse-type gastric cancer. It strongly predisposes an individual to tumor invasion and metastasis. E-cadherin dysfunction most often results from genetic CDH1 mutations, although epigenetic alterations may also result in protein dysfunction (Riquelme et al. 2015). E-cadherin interacts with the multifunctional mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NF-κB), and Rho-GTPase signaling pathways. In particular, RhoA mutations commonly accompany diffuse-type tumors. RhoA mutations may also result in impaired anoikis, a form of programmed cell death occurring in anchored cells after detaching from the surrounding extracellular matrix (ECM) (Tan and Yeoh 2015). Evasion of anoikis may contribute to the metastatic potential of diffuse-type gastric cancer. Diffuse-type histology is overrepresented among genomically stable gastric cancers and also associates with EBV positivity (Cancer Genome Atlas Research Network 2014).

Intestinal-type tumors are preceded by the Correa pathway, often with a contributing *H. pylori* infection. Recent studies found that distinct genetic changes also associate with intestinal-type tumors, most notably DNA hypermethylation and adenomatous polyposis coli (APC), B-cell lymphoma 2 protein (Bcl-2) K-Ras, and β-catenin mutations (Riquelme et al. 2015). Genomic instability precedes mutations and DNA methylation in intestinal-type gastric cancer (Cancer Genome Atlas Research Network 2014).

Mutations of TP53, p16, p21, B-Raf, and protein kinase B commonly accompany both histological subtypes.

6.9.2 Genomic instability and DNA methylation

The underlying genomic instability in gastric cancer may originate from either chromosomal or microsatellite instability. Microsatellite instability (MSI) seems to associate with a better prognosis and originates from dysfunctional mismatch repair systems (Polom et al. 2018b; Ratti et al. 2018). The mismatch repair system identifies base-pair mismatches in the DNA during cell replication, excises them, and resynthesizes the corresponding DNA sequence. Flaws in the system lead to an
increased mutation rate. Approximately 15% of gastric cancers associate with defects in the mismatch repair system (Bacani 2005).

The causes of chromosomal instability (CIN) remain contested, although the phenomenon features major alterations of the karyotype, including structural changes and dysploidy (Maleki and Röcken 2017). Approximately 85% of gastric cancers show a CIN.

DNA methylation is a central epigenetic mechanism in gastric carcinogenesis and the methylation phenotype is a promising molecular biomarker. Aberrant DNA methylation can be generally divided into two groups: global hypomethylation and regional hypermethylation (Tahara and Arisawa 2015). Global DNA hypomethylation appears to induce a genomic instability. Regional hypermethylation, occurring preferentially in promoter CpG islands, may efficaciously disable tumor suppressors (Calcagno et al. 2013). Hypermethylation is also seen in \textit{H. pylori}–infected benign gastric mucosa, which carries a heightened risk of developing cancer. The eradication of \textit{H. pylori} appears not to completely return DNA methylation to normal levels.

6.9.3 Oncogenes and tumor suppressors

The dysfunction of tumor suppressor p53, encoded by the TP53 gene, is a key alteration in gastric carcinogenesis. A dysfunctional p53 is incapable of halting mitosis upon detection of faulty DNA and guiding the cell to apoptosis (Fenoglio-Preiser et al. 2003). The function of p53 may be disrupted either by TP53 DNA methylation or through the loss of heterozygosity. Mutations in TP53 have been reported in nearly 80% of gastric cancers. Moreover, TP53 mutations appear to generalize with disease progression, although p53 dysfunction has also been recorded among precancerous foci (Karaman et al. 2010; Bellini et al. 2012).

Elevated prostaglandin E2 (PGE2) levels occur in the majority of gastrointestinal tumors, including gastric cancer (Huang and Chen 2011). PGE2 enhances proliferation, suppresses apoptotic signaling, and promotes invasiveness.

The multifunctional cytokine transforming growth factor beta (TGF-β) has a heterogenic function in gastric cancer. In early gastric carcinogenesis, it may function as a tumor suppressor, although in later events it may participate in oncogenic signaling (Mishra et al. 2005; Wu et al. 2010). In a clinical context, TGF-β1 serum levels were higher among patients with gastric cancer than among healthy
controls, and an elevated serum TGF-β1 level associated with venous invasion in gastric cancer (Lin et al. 2006).

Nuclear factor kappa B (NF-κB) is a multifunctional gene regulator protein affecting proliferation, cell fate determination, and inflammatory responses (Baud and Karin 2009). Expressed in gastric cancer cells, NF-κB can regulate transcriptional programs in response to cytokines, reactive oxygen species, and pathogenic antigens (Tian and Brasier 2003; Gilmore 2006). Prognosis worsens among patients with gastric cancer with a high tissue NF-κB expression compared to those with a low expression (Yamanaka et al. 2004).

The MAPK signaling pathway consists of a chain of kinases, including Ras, Raf, MEK, and MAPK (Orton et al. 2005). The signal is transmitted from the cell surface to the nucleus via a sequence of phosphorylations, switching kinases between active and inactive configurations. MAPK signaling regulates the cell cycle, differentiation, proliferation, and survival. In particular, K-Ras and B-Raf mutations seem particularly relevant in gastric cancer (Castellano and Santos 2011).

The epidermal growth factor (EGF) is an important effector in cell proliferation, motility, angiogenesis, and apoptosis (Tomas et al. 2014). Aberrant EGF signaling is common in malignant disease, including gastric cancer. EGF binds to the epidermal growth factor receptor (EGFR), which serves as an unfavorable prognostic biomarker and serves as a potential target for specific therapy in gastric cancer (Higaki et al. 2016).

Other gastric cancer–associated molecular mechanisms include dysfunction in the protein kinase B, inactivated adenomatous polyposis coli (APC) protein, flaws in apoptosis regulation by the B-cell lymphoma 2 (Bcl-2) protein, dysfunctional cell cycle regulation by p21, and VEGF overexpression (Shi et al. 2014). Among the epigenome, in addition to DNA methylation, histone modifications and ribonucleic acid (RNA) editing play a central role in gastric carcinogenesis (Padmanabhan et al. 2017).

### 6.9.4 Molecular classification of gastric adenocarcinoma

A novel molecular classification has been recently proposed by the Cancer Genome Atlas Research Network (2014). This classification introduces four gastric cancer subtypes, each with a specific molecular trait: chromosomal instability (CIN), microsatellite instability (MSI), Epstein–Barr virus positivity (EBV), and genetic
stability (GS). This classification is promising, although still unsuitable for routine use. Figure 9 presents the key features of each subtype. These consensus molecular subtypes provide a comprehensive basis for further studies and exploration aimed at developing a clinically applicable molecular classification for gastric cancer.

![Diagram showing gastric cancer subtypes](image)

**Figure 9.** Consensus molecular subtypes of gastric cancer with their key features. Reproduced with permission from *Springer Nature* (Cancer Genome Research Network 2014).

### 6.9.5 Invasion and metastasis

Invasion and the ability to metastasize are hallmarks of cancer. Upon the initiation of invasion, active tumor metastatic genes must outweigh the effect of metastatic suppressor genes in order for the cells to acquire any invasive potential. Alongside dysfunction in the adhesion proteins, an enhanced tissue degrading capability, and an enhanced motility, the cells may penetrate the basement layer and migrate into the surrounding tissue (Conlon and Murray 2019). Cancer cells can acquire an enhanced tissue degrading capability by expressing proteases. Subsequently, the malignant cells may continue to invade blood or lymphatic vessels resulting in metastasis.
Interactions with adjacent stromal cells result in an arrest to the malignant cells and in an extravasation at the metastatic site (Jiang et al. 2015). Alternatively, cancer cells can spread along the walls of the cavity they reach.

6.9.5.1 Matrix metalloproteinases (MMPs) and PROX1

The expression of several proteases has been widely studied in gastric cancer. Specifically, matrix metalloproteinases (MMPs) have proved quite promising in untangling the pathogenesis of gastric cancer (Sampieri et al. 2013; Jiang et al. 2015; Conlon and Murray 2019).

MMPs are inflammation-associated zinc-containing endopeptidases (Curran and Murray 1999). A total of 26 MMPs have been identified, distinguishable from other proteases given their dependence on a metal-ion cofactor. MMPs degrade the ECM, and are involved in the invasion and formation of metastases (Egeblad and Werb 2002). Additionally, MMPs cleave a variety of cell surface receptors and regulate cyto- and chemokine signaling (Van Lint and Libert 2007). In addition to the expression of MMPs, the dysregulation between MMPs and their inhibitors, tissue inhibitors of metalloproteinases (TIMPs), have proved relevant to cancer progression (Jackson et al. 2017; Gershtein et al. 2018; Laitinen et al. 2018; Böckelman et al. 2018).

Matrix metalloproteinase 14 (MMP14) is a membrane-bound MMP with strong ECM degrading capabilities. Also referred to as membrane-type 1 matrix metalloproteinase (MT1-MMP), emphasis is placed on its transmembrane position. Furthermore, tethered to the cell surface, a high MMP14 expression creates a proteolytic jacket, thereby degrading tissue barriers and, thus, aiding in the invasion and metastasis of several cancers (Castro-Castro et al. 2016; Turunen et al. 2017). However, sometimes the enzyme can be found in systemic circulation as a result of shedding from the membrane. The functions of membrane-type MMPs are not merely limited to the guiding mechanical forces, but may also alter signaling through the degradation of receptors and growth factors. Thus, membrane-type MMPs, including MMP14, are regarded as signaling proteases of the tumor microenvironment.

A high expression of MMP14 appears to associate with a poor outcome and increased metastasis in gastric cancer (He at al. 2013; Peng et al. 2013; de la Peña et al. 2014; Dong et al. 2015; Naseh et al. 2016). Previous research on MMP14 in gastric cancer has primarily focused on tissue sections with only a few studies on
circulating MMP14 published thus far. For instance, Mimori et al. (2008) discovered that a high MMP14 gene expression in peripheral blood associated with advanced disease. *In vivo* and *in vitro* laboratory studies of MMP14 in gastric cancer have also confirmed that cells with a high MMP14 expression show an enhanced invasive and metastatic behavior (Nonaka et al. 2005; Zheng et al. 2013).

The regulation of MMP14 synthesis is context-dependent and may be influenced by various factors within the tumor microenvironment (Turunen et al. 2017). Prospero homeobox protein 1 (PROX1) is a transcription factor with physiological functions related to organ development and cell fate determination, but which also appears to contribute to carcinogenesis in several cancers (Wigle et al. 1999; Wigle and Oliver 1999; Risebro et al. 2009; Skog et al. 2011; Elsir et al. 2012). The reduction of PROX1 levels decelerate gastric cancer growth *in vitro* (Zhang et al. 2016). Gramolelli et al. (2008) found that PROX1 inhibits the transcription of MMP14 by directly binding to its promoter region. The link between MMP14 and PROX1 was examined in murine models and in the cell lines of several cancer types revealing concordant results, although gastric cancer was not included. Studies on the prognostic significance of PROX1 tissue expression have yielded conflicting results. Its high tissue expression evaluated using immunohistochemistry has been connected to favorable as well as unfavorable prognoses (Park et al. 2017; Laitinen et al. 2017; Ueta et al. 2018).

### 6.9.5.2 Trypsin proteases

Trypsin and its specific inhibitor, pancreatic secretory trypsin inhibitor (PSTI) — also known as serine protease inhibitor Kazal-type 1 (SPINK1) — are found in physiological conditions through pancreatic secretions and elevated, imbalanced levels in pancreatitis (Pubols et al. 1974; Haglund et al. 1986; Liddle 2004). PSTI is also found in the gastrointestinal tract mucosa protecting the epithelial cells from enzymatic destruction (Stenman et al. 1991).

Elevated trypsin and PSTI levels have been identified among patients with malignant disease, while in the context of malignant diseases the enzyme inhibitor is also referred to as tumor-associated trypsin inhibitor (TATI). TATI was first identified from the urine of a patient with a gynecological malignancy and subsequent studies showed that its elevated level served to distinguish patients with a malignant gynecological disease from those with benign disease (Huhtala et al. 1982; Huhtala et al. 1983).
Elevated TATI levels have also been discovered among patients with infectious or inflammatory disease and resulting from a tissue injury (Paavonen et al. 1989; Lehtovirta et al. 1990). Thus, TATI appears to also function as an acute-phase reactant, such that its levels resemble that of other acute-phase reactants, including the C-reactive protein (CRP) and ferritin (Ogawa 1988; Solakidi et al. 2004). Among patients with gastric cancer, a high tissue TATI expression appears to associate with a low cancer staging and accompany a better prognosis, while high serum TATI levels associate with a higher cancer stage and the presence of metastasis (Wiksten et al. 2005; Kemik et al. 2013). The loss of TATI expression has also been studied at the protein and RNA levels, whereby lower levels associate with an unfavorable prognosis (Wiksten et al. 2008; Lei et al. 2012).

A high TATI level often accompanies a high level of tumor-associated trypsin (Stenman et al. 1991; Itkonen and Stenman 2014). Tumor-associated trypsin-1 and tumor-associated trypsin-2 (TAT-2) (which represents a major component) serve as the target proteolytic enzymes for TATI and can promote carcinogenesis by facilitating invasiveness via activating pro-urokinase and MMPs (Sorsa et al. 1997; Moilanen et al. 2003). On the one hand, TATI can suppress invasiveness by inhibiting trypsin; but, on the other hand, it can promote carcinogenesis through its growth factor capabilities, including EGF receptor activation (Ozaki et al. 2009; Itkonen and Stenman 2014). Additionally, TATI appears to modulate apoptosis in certain cancers (including hepatocellular, breast, and colorectal cancers), further complicating its signaling in cancer (Räsänen et al. 2016).

TAT-2 is expressed among patients with various malignancies, including gastric cancer (Koivunen et al. 1991: Koshikawa et al.1992; Miyata et al. 1999; Stenman 2016). Concordant with its carcinogenic capabilities, its high expression seems to associate with an increased invasiveness of gastric cancer cells (Kato et al. 1998). Among patients with gastric cancer, a high TAT-2 expression associated with diffuse gastric cancer, suggesting a poor prognosis (Ichikawa et al. 2000)

### 6.9.5.3 Other proteases

Other proteases involved in cancer invasion include cysteine cathepsins, serine proteinases, and heparinase. Cysteine cathepsins disrupt the tissue architecture, promote extravasation, and may participate in the formation of neovasculature (Olson and Joyce 2015). Similarly, serine proteases contribute to ECM degradation, but may also activate MMPs and induce prostaglandin synthesis (Soreide et al. 2006). A high heparinase expression is found in a variety of cancers associated with
an increased invasiveness and metastasis (Jin and Zhou 2017). This enzyme is also capable of releasing ECM-bound growth factors (Sanderson et al. 2017).

6.10 Inflammation and cancer

Immune cells have long been found to infiltrate tumor tissues; today, it is clear that nearly all solid tumors contain immune cells (Pagès et al. 2010). Historically, inflammatory cells infiltrating into malignant tumors were thought to stem from the immune system in an attempt to eradicate a tumor. However, today we understand that inflammatory cells can also participate in carcinogenesis such as through the release of free radicals and the promotion of genomic instability and mutability (Grivennikov et al. 2010). An increased risk of cancer has been found both among immunosuppressed patients and among patients with autoimmune or other chronic inflammatory disease (Vajdic and van Leeuwen 2009; Francesccone et al. 2015). Extensive leukocyte infiltration in colorectal and gastric tumors appears to associate with a favorable outcome, although tumor-infiltrated immune cells as also associate with an unfavorable prognosis in certain cancers, such as breast cancer and melanoma (Leek et al. 1996; Coca et al. 1997; Ishigami et al. 2000; Ribatti et al. 2003). The immune system consists of precisely coordinated interactions between the two immunological arms: the innate and the adaptive arms. Table 6 summarizes the cells from the innate and adaptive immunity with examples of tumorigenic and antitumor functions.

Table 6. Innate and adaptive immune cells with examples of their tumorigenic and antitumor functions (Caruana et al. 2018; Yang and Lin 2017; Hanahan and Coussens 2012; Grivennikov et al. 2010).

<table>
<thead>
<tr>
<th>Innate immunity</th>
<th>Antitumor function</th>
<th>Tumorigenic function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>N1: Direct cytotoxicity, regulation of CTLs</td>
<td>N2: Cytokine, protease, ROS production</td>
</tr>
<tr>
<td>Natural killer (NK) cells</td>
<td>Direct cytotoxicity, cytokine production</td>
<td></td>
</tr>
<tr>
<td>Macrophages, dendritic cells (DCs)</td>
<td>M1 and dendritic cells: Antigen presentation, IL-12, type 1 IFN production</td>
<td>M2: Suppression of CTLs and NK cells, CSF-1 production</td>
</tr>
<tr>
<td>Myeloid-derived suppressor cells (MDSCs)</td>
<td>Suppression of CTLs, production of proteases, ROS, and TGF-β</td>
<td></td>
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<tr>
<td>Mast cells</td>
<td>Suppression of CTLs and NK cells, cytokine production</td>
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<tr>
<td>Adaptive immunity</td>
<td>Antitumor function</td>
<td>Tumorigenic function</td>
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<tr>
<td>CD8+ Cytotoxic T cells (CTLs)</td>
<td>Direct cytotoxicity, antitumor cytokine production</td>
<td>Production of growth-promoting cytokines</td>
</tr>
<tr>
<td>CD4+ Th1 cells</td>
<td>CTL regulation, pro-apoptotic cytokine production</td>
<td>Production of growth-promoting cytokines</td>
</tr>
<tr>
<td>CD4+ Th2 cells</td>
<td>CTL suppression, M2 macrophage and B cell regulation</td>
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<tr>
<td>CD4+ Th17 cells</td>
<td>CTL activation</td>
<td>Production of growth-promoting cytokines</td>
</tr>
<tr>
<td>CD4+ Treg cells</td>
<td>Suppression of tumorigenic cytokines</td>
<td>Immunosuppression</td>
</tr>
<tr>
<td>γδ T cells</td>
<td>Direct cytotoxicity, indirect stimulation of CTLs</td>
<td>Immunosuppression, suppression of DC maturation</td>
</tr>
<tr>
<td>B cells</td>
<td>Tumor-specific antibody production, antigen presentation</td>
<td>Immunosuppression, mast cell activation, cytokine production</td>
</tr>
</tbody>
</table>

Abbreviations: CTL, cytotoxic T cell; NK, natural killer cell; MDSC, myeloid-derived suppressor cell; IL, interleukin; IFN, interferon; ROS, reactive oxygen species; TGF-β, transforming growth factor β; DC, dendritic cell; Th1, T-helper type 1 cell; Th2, T-helper type 2 cell; Th17, T-helper type 17 cell.

### 6.10.1 Innate immunity

Tumor-associated macrophages (TAMs) account for a considerable portion of the leukocyte population within malignant lesions and consist of antitumor M1 and tumorigenic M2 subtypes (Table 6). M2-type TAMs are particularly relevant in the promotion of tumorigenesis through participation in tumor invasion and metastasis through the secretion of colony-stimulating factor 1 (CSF-1), EGF, and VEGF (Condeelis and Pollard 2006; Yang and Lin 2017). M1-type TAMs and dendritic cells (DCs) function as antigen-presenting cells and secrete antitumor cytokines.

Other cells with innate immunity within the tumor microenvironment include most importantly tumor-associated neutrophils (TANs) and myeloid-derived suppressor cells (MDSCs). TANs consist of antitumor N1 and tumorigenic N2 subtypes (Table 6). Their functions are governed by the available effectors, importantly, TGF-β signaling steers N2-type TANs towards producing tumorigenic cytokine- and chemokines (Sagiv et al. 2015; Shaul and Fridlender 2017). MDSCs secrete reactive oxygen and nitrogen species, proteases, TGF-β, and can inhibit cytotoxic T lymphocytes (Gabrilovich and Nagaraj 2009). The deletion of MMP9 from MDSCs apparently cancels their tumorigenic potential in vitro (Yang et al. 2004). Natural
killer (NK) cells mostly carry antitumor properties, whereas mast cells appear primarily tumorigenic. Among granulocytes, eosinophils and basophils play less important roles in tumorigenesis compared to neutrophils.

6.10.1.1 Toll-like receptors (TLRs)

Toll-like receptors (TLRs) are a family of pattern-recognition receptors, situated transmembranously on cell surfaces (Akira et al. 2006). We know of a total of 13 TLRs, of which 10 are found in humans, each of which binds to distinct antigens. TLRs widely express on the cells with an innate immunity, since their activation is vital to the function of antigen-presenting cells, such as macrophages and DCs. TLRs appear to also play important roles in malignant diseases, delivering signals between tumor cells, the tumor microenvironment, and its resident leukocytes. In pathogen recognition, TLRs are activated by pathogen-associated molecular patterns (PAMPs), such as bacterial lipopolysaccharide. In the context of malignant diseases, however, TLRs can be activated by damage-associated molecular patterns (DAMPs), such as cellular debris including cytosolic and nuclear components (West and Jenkins 2015; Hernandez et al. 2016).

Due to their physiological role in regulating inflammation, TLRs have long proved promising and interesting as a family of proteins to disentangle the pathology behind inflammation-associated cancer. Studies of the TLR role in malignant diseases have, however, yielded discordant results, showing that they may context dependently participate in either promoting or suppressing cancer development (Pradere et al. 2014). On the one hand, TLRs may facilitate tumorigenesis through the activation of COX-2/PGE2 axis and, on the other hand, stimulate secretion of the tumor-suppressive type I interferon from DCs.

TLRs are of specific interest in the context of gastric cancer since they are activated by the gastric cancer–predisposing pathogen *H. pylori*. *H. pylori* induces heterogenous TLR expression and distinct TLR gene variants may predispose an individual to gastric carcinogenesis by altering immune responses against neoplastic cells (Smith 2014; Pachathundikandi et al. 2015; Ravishankar Ram et al. 2015; Xu et al. 2017). However, the function of TLRs in gastric cancer is not restricted to the interplay with *H. pylori*. For instance, Zhao et al. (2019) recently demonstrated that TLR2 expression is linked to patient prognosis in gastric cancer independent of *H. pylori* colonization of the stomach.
TLR4 polymorphisms predispose an individual to gastric cancer and its activation by bacterial lipopolysaccharide apparently links to increased metastasis (Castañó-Rodríguez et al. 2013; Zhou et al. 2014). Pimentel–Nunes et al. (2011) studied TLR4 levels, along with TLR2 and TLR5, among different grades of gastric mucosal lesions ranging from healthy mucosa to invasive cancer, identifying a correlation between a high TLR expression and further progressed mucosal lesions. However, TLR expression was highest among dysplastic lesions rather than among invasive cancer, suggesting that TLR activation may be of particular importance in the onset of invasion. Furthermore, high TLR3 and TLR2 expressions have been linked to an unfavorable prognosis and the presence of metastasis in gastric cancer (Fernandez-Garcia et al. 2014; Yang et al. 2014). Finally, TLR5 activation by bacterial flagellin increased the proliferation of gastric cancer cells, whereas TLR7 agonism by imiquimod, a specific agonist, reduced proliferation (Park et al. 2011; Jiang et al. 2016).

6.10.2 Adaptive immunity

Adaptive immunity effectively eliminates pathogens and forms the immunological memory. Cells related to adaptive immunity within malignant tumors are primarily composed of cytotoxic T lymphocytes (CTLs), T-helper type 1 (Th1), type 2 (Th2), and type 17 (Th17) lymphocytes, regulatory T cells, γδ T cells, and B cells (Table 6).

The role of lymphocytes in the inflammatory tumor microenvironment is complex and several anti- and procarcinous mechanisms have been identified. Antitumor mechanisms include direct and indirect cytokine-mediated cancer cell destruction by cytotoxic T cells and γδ T cells (Caruana et al. 2018; Yang and Lin 2017). The abundance of cytotoxic T cells within several tumor types has been linked to a better prognosis (Pagès et al. 2010). Other antitumor mechanisms include CTL activation by Th1 cells and the production of antitumor antibodies by B cells. Tumorigenic mechanisms of the adaptive immunity include CTL suppression by Th2 and regulatory T cells, M2-type TAM activation by Th2 lymphocytes, and the release of tumorigenic cytokines by regulatory T and Th17 cells.

The programmed cell death 1 (PD1) protein functions as an immune checkpoint to distinguish foreign cells (Chamoto et al. 2017). PD1 is expressed on activated T and B lymphocytes and binds to programmed cell death ligands (PDLs), which are expressed extensively in the body. Managing PD1/PDL signaling is a potent therapeutic route in cancer given that increasing evidence has emerged indicating that aberrant PD1 is a major cancer immune escape mechanism (Iwai et al. 2017).

50
Cytotoxic T lymphocyte–associated protein 4 (CTLA4) is another immune checkpoint protein, functioning as a T-cell brake (Sharma and Allison 2015). Humoral CTLA4 blockage enables lymphocytes to more effectively engage foreign cells, such as cancer cells. James P. Allison and his colleagues conducted the first studies regarding CTLA4 blockage in the 1990s, resulting in complete tumor eradication in mice (Leach et al. 1996). James P. Allison and Tasuku Honjo jointly received the Nobel Prize in Physiology or Medicine in 2018 for their studies on immunotherapy in cancer: Allison for his work with CTLA4 and Honjo for his investigation on PD1/PDL.

6.10.3 The tumor microenvironment and the immune surveillance hypothesis

Within infection sites, the inflammatory microenvironment persists transiently compared to its chronic persistence within tumors. The tumor stroma, the parenchyma, and their effectors form the tumor microenvironment (Hanahan and Weinberg 2011; Yang and Lin 2017; Figure 10).

![Figure 10. Key cells of the tumor microenvironment. Red: predominantly tumorigenic; yellow: predominantly tumorigenic but with antitumor potential; green: predominantly antitumor. Abbreviations: CAF, cancer-associated fibroblast; MDSC, myeloid-derived suppressor cell; Treg, regulatory T cell; Th2, T-helper cell type 2; Th17, T-helper cell type 17; M, macrophage; N, neutrophil; NK, natural killer cell; Th1, T-helper type 1 cell; CTL, cytotoxic T cell; DC, dendritic cell.](image)

The tumor stroma contains fibroblasts, pericytes, epithelial cells, mesenchymal cells, and inflammatory cells. It contributes to the changing local microenvironment along the actual neoplastic cells ultimately enabling invasion and metastasis (Giaccia and
Schipani 2010; Sun et al. 2014). Upon metastasis, a new malignant tissue microenvironment must be configured at the metastatic site to support the still sparse cancer cells. Some sites may natively possess a propitious environment or may form from factors released to systemic circulation by the primary tumor (Aguado et al. 2017; Celià-Terrassa and Kang 2018). The native or induced prometastatic sites are called metastatic niches. A wide array of signaling molecules operate within the tumor microenvironment, including numerous proteases, growth factors, inflammatory signaling factors, and other cytokines and chemokines.

The theory of immune surveillance presupposes that cells are constantly overlooked by the immune system (Swann and Smyth 2007). The immune system attempts to detect sparse transformed cells and destroy them before further propagation. In order to keep proliferating, transformed cells must evade the immune surveillance by disguising themselves entirely from the immune system or through heavy immunosuppression. This theory is often depicted in three stages: during ‘elimination’, the immune system successfully eradicates transformed cells; during ‘equilibrium’, tumors successfully evade immunological eradication to some extent and may become clinically apparent; and during ‘escape’, the immune system fails to eradicate the transformed cells and without treatment tumorigenesis progresses (Figure 11). Similar mechanisms also apply to the formation of metastases.

**Figure 11.** Schematic representation of the immunosurveillance hypothesis. Reproduced with permission from the American Association of Immunologists (Finn 2018).
6.10.4 Systemic inflammation

In addition to local events, a systemic inflammatory reaction distinguished by an elevated blood C-reactive protein (CRP) level or by an elevated blood neutrophil count is sometimes seen among patients with malignant disease (Allin and Nordestgaard 2011). A systemic inflammatory reaction serves as an unfavorable prognostic factor in several cancers, including gastric cancer (Liu et al. 2017; Wang et al. 2017b).

The Glasgow prognostic score (GPS) and the modified Glasgow prognostic score (mGPS) combine information on the CRP and albumin levels. A high GPS/mGPS serves as an unfavorable prognostic factor in several cancers, including gastric cancer (Simmons et al. 2017; Gao and Huang 2014). In GPS, patients are given a score from 0 to 2. A plasma CRP over 10 mg/l and an albumin under 35 g/l both yield a score of 1. mGPS differs from GPS by scoring patients with an albumin <35 g/l as 0 if CRP is simultaneously <10 mg/l. GPS/mGPS represents a promising prognostic score, although it is not yet routinely used in gastric cancer.

6.10.4.1 C-reactive protein (CRP)

C-reactive protein (CRP) is a polymeric acute-phase reactant produced by hepatocytes in response to infection, inflammation, and external tissue damage. Its physiological function is to activate complement C1q by binding lysophosphatidylcholine. Today, it is primarily clinically used to follow the course of an infection (Thompson et al. 1999).

CRP levels have been studied among patients with various cancers, including colorectal, pancreatic, and gastric cancers, and an elevated level typically associates with an adverse prognosis (Chang et al. 2010; Nozoe et al. 2011; Yu et al. 2013; Kersten et al. 2013; Salmiheimo et al. 2016; Køstner et al. 2016).

An elevated preoperative CRP level appears to serve both as a diagnostic and prognostic biomarker in gastric cancer and associate with deeper tumors, the presence of metastasis, and a higher risk of recurrence (Chang et al. 2010; Nozoe et al. 2011). A meta-analysis by Yu et al. (2013), consisting of 2597 patients, found that CRP serves as an unfavorable prognostic factor. Yet, in 3 of 12 original studies no connection between CRP and prognosis was found. The authors argued that the number of original studies included was quite limited given the lack of relevant high-quality studies on the subject.
6.10.4.2 Cytokines and chemokines

Tumor necrosis factor alpha (TNFα) participates in the regulation of the immune system, cell proliferation, differentiation, survival, and apoptosis. TNFα was initially named for its antitumor properties, but later discoveries indicated that it actually promotes tumorigenesis (Sethi et al. 2008). In addition, TNFα is produced by both immune cells and neoplastic cells, and affects the expression of several inflammatory effectors (Anderson et al. 2004). TNFα is regarded as a key molecule in inflammation-associated cancer and affects nearly all stages of carcinogenesis from the development of a primary tumor to late metastases.

Interleukins (ILs) were first recognized as leukocyte-signaling molecules. Today, more than 50 functional ILs have been identified and several represent important effectors within the tumor microenvironment (Brocker et al. 2010). IL-1, IL-4, and IL-6 regulate proliferation, invasion, angiogenesis, and apoptosis via oncogenic NF-κB, MAPK, and STAT3 signaling, whereas IL-2, IL-15, and IL-21 promote cytotoxic T and NK cell–mediated tumor cell destruction (Dmitrieva et al. 2016; Setrrerahmane and Xu 2017). IL-6 inhibition has yielded promising antitumor effects in a variety of malignancies (Guo et al. 2012; Kampan et al. 2018).
7 AIMS OF THE STUDY

This study aimed to determine the expression and prognostic significance of several inflammation-related biomarkers in gastric cancer.

The specific study aims were related to evaluating the prognostic value of:

I MMP14 serum levels in relation to clinicopathological parameters.

II the MMP14 and PROX1 relationship in gastric cancer tissue expression in relation to clinicopathological parameters.

III TLR1, TLR2, TLR4, TLR5, TLR7, and TLR9 gastric cancer tissue expression levels in relation to clinicopathological parameters.

IV TAT-2 and TATI serum levels, the TAT-2/TATI ratio, and preoperative plasma CRP in relation to clinicopathological parameters.
8 PATIENTS AND METHODS

8.1 Patients (studies I–IV)

The cohort consisted of 313 consecutive patients operated on for histologically proven gastric adenocarcinoma in the Department of Surgery, Helsinki University Hospital between 2000 and 2009. The exclusion criteria included synchronous cancers and a previous history of malignant diseases. Out of the 313 patients, 152 (48.6%) were male with a median age of 67.4 years (interquartile range [IQR], 57.1–76.5). Disease-specific 5-year survival across the entire cohort reached 43.3%, with a median survival of 3.0 years. In total, 228 (72.8%) patients were operated on with a curative intent, while the remainder underwent palliative treatment. The seventh version of the TNM classification was used to determine cancer staging (Sobin et al. 2009). Among 124 (39.6%) patients, the cancer was of an intestinal type (Lauren 1965). A subtotal gastrectomy was performed on 160 (51.1%) patients, whereas total gastrectomy was required in 153 (48.9%) cases. Disease was lymph node–positive in 198 (63.3%) patients, and distant metastases were present in 63 (20.1%) cases. A D1 lymph node dissection was performed on 107 (34.2%) patients, whereas 203 (64.9%) underwent a D2 or D2+ dissection. Preoperative chemotherapy was administered to 15 (4.8%) patients and 125 (39.9%) received postoperative chemotherapy. Table 7 provides an overview of the gastric cancer patient cohort.

In studies I and IV, circulating biomarker serum levels were compared across 240 patients with gastric cancer and 48 patients with benign disease (controls) undergoing surgery or gastroscopy between 2000 and 2012. Blood samples were drawn within 30 days (median, 1 day; range, 26 days) preoperatively. Among controls, 27 (56.3%) were male and the median age was 61.0 years (IQR, 55.0–71.1). Controls underwent surgery or gastroscopy for reasons including gastroesophageal reflux disease, hematemesis, esophageal hiatus hernia, duodenal polyps, duodenal perforation, peptic ulcer disease, benign gastric tumors, or other nonmalignant reason.

We updated survival data in September 2017 and in August 2019. Information was obtained from the Population Register Center of Finland, Statistics Finland, and patient medical records. All studies were approved by the Surgical Ethics Committee of Helsinki University Hospital (Dnro HUS 226/E6/ 06, extension TMK02 §66 17.4.2013). The National Supervisory Authority of Health and Welfare approved our
study of archived tissue samples without requiring specific individual consent (Valvira Dnro 10041/06.01.03.01/2012).

**Table 7.** Clinicopathological characteristics of gastric cancer patients (n = 313). Preoperative chemotherapy was administered to 15 (4.8%) patients.

<table>
<thead>
<tr>
<th>Clinicopathological variable</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No preoperative chemotherapy (pTNM)</td>
</tr>
<tr>
<td>Age &lt;67</td>
<td>145 (48.7)</td>
</tr>
<tr>
<td>Age ≥67</td>
<td>153 (51.3)</td>
</tr>
<tr>
<td>Men</td>
<td>145 (48.7)</td>
</tr>
<tr>
<td>Women</td>
<td>153 (51.3)</td>
</tr>
<tr>
<td>Treatment with curative intent</td>
<td>218 (73.2)</td>
</tr>
<tr>
<td>Palliative treatment</td>
<td>80 (26.8)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>60 (20.2)</td>
</tr>
<tr>
<td>II</td>
<td>69 (23.2)</td>
</tr>
<tr>
<td>III</td>
<td>108 (36.4)</td>
</tr>
<tr>
<td>IV</td>
<td>60 (20.2)</td>
</tr>
<tr>
<td>Tumor classification</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>48 (16.1)</td>
</tr>
<tr>
<td>T2</td>
<td>42 (14.1)</td>
</tr>
<tr>
<td>T3</td>
<td>91 (30.5)</td>
</tr>
<tr>
<td>T4</td>
<td>117 (39.3)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>98 (34.1)</td>
</tr>
<tr>
<td>N1</td>
<td>43 (15.0)</td>
</tr>
<tr>
<td>N2</td>
<td>68 (23.7)</td>
</tr>
<tr>
<td>N3</td>
<td>78 (27.2)</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>238 (79.9)</td>
</tr>
<tr>
<td>M1</td>
<td>60 (20.1)</td>
</tr>
<tr>
<td>Laurén classification</td>
<td></td>
</tr>
<tr>
<td>Intestinal</td>
<td>119 (39.9)</td>
</tr>
<tr>
<td>Diffuse</td>
<td>179 (60.1)</td>
</tr>
<tr>
<td>Location</td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>16 (5.4)</td>
</tr>
<tr>
<td>Middle</td>
<td>128 (43.3)</td>
</tr>
<tr>
<td>Distal</td>
<td>123 (41.5)</td>
</tr>
<tr>
<td>Widespread</td>
<td>29 (9.8)</td>
</tr>
</tbody>
</table>


8.2 Immunohistochemistry (studies II and III)

In studies II and III, we studied MMP14, PROX1, TLR1, TLR2, TLR4, TLR5, TLR7, and TLR9 expression levels in tissue by immunohistochemistry using specific antibodies.

8.2.1 Archived tissue samples and immunohistochemical staining

Figure 12 provides a schematic representation of the immunohistochemical staining procedure. First, each sample was given an identification number for anonymous analysis. Subsequently, 4 1.0-mm tissue cores were taken from each tumor sample and embedded in a new paraffin block, resulting in tissue microarrays (TMAs) (Kononen et al. 1998). An automatic tissue microarrayer was used to process the samples (TMA Grand Master, 3D Histech Ltd, Budapest, Hungary). Then, the TMAs were cut into 4-μm sections and fixed onto slides (stored at 37°C for 12–24 h) for immunohistochemical staining. The procedure was continued using deparaffinization in xylene and rehydration to lower the concentrations of ethanol and distilled water. The slides were then prewarmed in a PT module (LabVision UK Ltd, UK) to 65°C and, subsequently, incubated for 20 min at 98°C for antigen retrieval (Tris-EDTA buffer, pH 9.0 or Tris-HCl buffer, pH 8.5). The immunohistochemical staining was performed in an Autostainer 480 (LabVision) using the Dako detection system [Dako REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse (Dako, Glostrup, Denmark)] at room temperature. Endogenous peroxidases were blocked using 0.3% Dako REAL Peroxidase-Blocking Solution, and incubated at 5 min. The slides were then incubated with the primary antibody. Table 8 summarizes the primary antibodies used, their dilutions, and incubation times. Subsequently, the slides were incubated with the peroxidase-conjugated Dako REAL EnVision/HRP, Rabbit/Mouse (ENV) secondary antibody for 30 min and visualized using incubation with the Dako REAL DAB+ Chromogen for 10 min, followed by counterstaining with Meyer’s hematoxylin. For the PROX1 staining, the detection system was different (ImmPRESS HRP Polymer Detection Kit, Peroxidase, anti-goat IgG, Vector Laboratories, Burlingame, CA, USA). Finally, samples were washed in water for 10 min and then mounted in Aquamount (BDH, Poole, UK). Between each step of the staining procedure, slides were washed with phosphate buffered saline (PBS)-0.04%-Tween20.
Figure 12. A schematic representation of the immunohistochemical staining procedure.
Table 8. Overview of the primary antibodies used in immunohistochemistry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Dilution</th>
<th>Incubation</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP14 mAb</td>
<td>Chemicon International, Temecula, CA, USA</td>
<td>1:70</td>
<td>1 h (RT)</td>
<td>II</td>
</tr>
<tr>
<td>PROX1 pAb</td>
<td>R&amp;D Systems, Minneapolis, MN, USA</td>
<td>1:1800</td>
<td>ON (RT)</td>
<td>II</td>
</tr>
<tr>
<td>TLR1 pAb</td>
<td>Santa Cruz Biotechnology, Dallas, TX, USA</td>
<td>1:100</td>
<td>1 h (RT)</td>
<td>III</td>
</tr>
<tr>
<td>TLR2 pAb</td>
<td>Santa Cruz Biotechnology, Dallas, TX, USA</td>
<td>1:200</td>
<td>ON (RT)</td>
<td>III</td>
</tr>
<tr>
<td>TLR4 mAb</td>
<td>Santa Cruz Biotechnology, Dallas, TX, USA</td>
<td>1:2000</td>
<td>1 h (RT)</td>
<td>III</td>
</tr>
<tr>
<td>TLR5 mAb</td>
<td>Novus Biologicals, Centennial, CO, USA</td>
<td>1:100</td>
<td>1 h (RT)</td>
<td>III</td>
</tr>
<tr>
<td>TLR7 pAb</td>
<td>Novus Biologicals, Centennial, CO, USA</td>
<td>1:500</td>
<td>1 h (RT)</td>
<td>III</td>
</tr>
<tr>
<td>TLR9 mAb</td>
<td>Santa Cruz Biotechnology, Dallas, TX, USA</td>
<td>1:300</td>
<td>ON (RT)</td>
<td>III</td>
</tr>
</tbody>
</table>

Abbreviations: pAb, polyclonal antibody; mAb, monoclonal antibody; ON, overnight; RT, room temperature.

8.2.2 Quantification of immunoreactivity

In studies II and III, we quantified the MMP14 and TLR expression levels in cancer cells by evaluating the cytoplasmic staining intensity in cancer cells: 3 indicated strong staining, 2 moderate staining, 1 weak staining, and 0 no staining. Among the 313 patient samples, 278 samples stained for MMP14 had cancer cells in them and were suitable for scoring. Correspondingly, 282 TLR1-stained, 275 TLR2-stained, 281 TLR4-stained, 277 TLR5-stained, 268 TLR7-stained, and 277 TLR9-stained samples included cancer cells and were suitable for scoring. The MMP14 and TLR expression levels were grouped as low (scores 0 and 1) and high (scores 2 and 3) expression groups for the final analyses (Figures 13 and 14).
Figure 13. Microscopy images from the immunohistochemistry of gastric cancer tumors with low and high cytoplasmic TLR immunoreactivities. Reproduced with permission from Springer Nature.
In study II, PROX1 expression was quantified by evaluating the proportion of PROX1-positive nuclei: 4 indicated over 75% PROX1-positive nuclei, 3 represented 50% to 75%, 2 represented 25% to 50%, 1 represented less than 25%, and 0 indicated no reactivity in the nuclei. For the final analyses, the nuclear PROX1 expression levels were grouped as PROX1-positive and PROX1-negative samples (Figure 14).

Among the four TMA spots extracted from each original tumor sample, the one with the highest score was chosen to represent the patient in the statistical analyses. All TMA spots were scored by two independent researchers, AK and JH (senior pathologist), both blinded to the clinical data. Cases with a discrepancy were re-evaluated together and the final scoring was reached through consensus.

**Figure 14.** Microscopy images of the immunohistochemistry of gastric cancer tumors with A) MMP14 immunoreactivity and B) PROX1 immunoreactivity. Reproduced with permission from Wiley.
8.3 Serum and plasma samples (studies I and IV)

In study I, a commercial MMP14 enzyme-linked immunosorbent assay (ELISA) kit was used (Human MMP14 ELISA kit, ab197747, Abcam, Cambridge, UK). Serially diluted standards were prepared using the sample diluent from the kit. In total, 50 μl of the prepared standards, undiluted samples, and the antibody mixtures were added to the precoated wells. The wells were then incubated on a plate shaker at room temperature for 1 h and, then, washed with water. Next, 100 μl of the provided substrate was added to each well, followed by 10-min incubation at room temperature. Finally, the reaction was stopped by adding the provided stop solution. For quantification, absorbance was recorded at 450 nm (Victor™ X4, PerkinElmer, Singapore), followed by a standard curve estimation. All of the substances and solutions used were provided in the kit. Figure 15 provides a schematic representation of the quantitative sandwich-type assay.

In study IV, the plasma CRP levels in patients with gastric cancer were determined using a high-sensitivity method. A time-resolved immunofluorometric assay (TR-IFMA) was applied to the microtitration plates with a monoclonal CRP antibody (anti-hCRP, code 6405, Medix Biochemica, Espoo, Finland), according to the manufacturer instructions. Similarly, TR-IFMA was applied to determine the serum TAT-2 and TATI levels (Osman et al. 1993).

![Diagram](image)

**Figure 15.** A schematic representation of the quantitative sandwich-type assay of the ELISA kit used. 1, immobilization antibody; 2, capture antibody; 3, HRP-conjugated detection antibody.
8.4 Cells (study II)

8.4.1 Cell culture

We used a total of 11 human gastric cancer cell lines (Sekiguchi et al. 1978; Barranco et al. 1983; Ochiai et al. 1985; Motoyama et al. 1986; Yashiro et al. 1995; Yokozaki 2000; Kato et al. 2010; Okuno et al. 2019; Table 9).

Table 9. Overview of the cell lines used in study II.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Extraction site</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MKN-7</td>
<td>Intestinal-type gastric adenocarcinoma</td>
<td>Lymph node metastasis</td>
<td>JCRB Cell Bank¹</td>
</tr>
<tr>
<td>MKN-28</td>
<td>Intestinal-type gastric adenocarcinoma</td>
<td>Liver metastasis</td>
<td>H. Yokozaki²</td>
</tr>
<tr>
<td>MKN-74</td>
<td>Intestinal-type gastric adenocarcinoma</td>
<td>Liver metastasis</td>
<td>JCRB Cell Bank¹</td>
</tr>
<tr>
<td>MKN-45</td>
<td>Diffuse-type gastric adenocarcinoma</td>
<td>Liver metastasis</td>
<td>JCRB Cell Bank¹</td>
</tr>
<tr>
<td>TMK-1</td>
<td>Diffuse-type gastric adenocarcinoma</td>
<td>Lymph node metastasis</td>
<td>H. Yokozaki²</td>
</tr>
<tr>
<td>NUGC3</td>
<td>Diffuse-type gastric adenocarcinoma</td>
<td>Skeletal muscle metastasis</td>
<td>JCRB Cell Bank¹</td>
</tr>
<tr>
<td>KATOIII</td>
<td>Diffuse-type gastric adenocarcinoma</td>
<td>Pleural effusion</td>
<td>JCRB Cell Bank¹</td>
</tr>
<tr>
<td>OCUM-12</td>
<td>Diffuse-type gastric adenocarcinoma</td>
<td>Ascites</td>
<td>M. Yashiro³</td>
</tr>
<tr>
<td>OCUM-14</td>
<td>Diffuse-type gastric adenocarcinoma</td>
<td>Ascites</td>
<td>M. Yashiro³</td>
</tr>
<tr>
<td>OCUM-2MD3</td>
<td>Diffuse-type gastric adenocarcinoma</td>
<td>Primary tumor</td>
<td>M. Yashiro³</td>
</tr>
<tr>
<td>AGS</td>
<td>Gastric adenocarcinoma</td>
<td>Primary tumor</td>
<td>ATCC⁴</td>
</tr>
</tbody>
</table>

¹Japanese Collection of Research Bioresources Cell Bank, National Institutes of Biomedical Innovation, Health and Nutrition, Osaka, Japan. ²Professor H. Yokozaki, Hiroshima University School of Medicine, Hiroshima, Japan. ³Professor M. Yashiro, Osaka City University Graduate School of Medicine, Osaka, Japan. ⁴American Type Culture Collection, Manassas, VA, USA.

The cells were cultured in standard conditions (37°C, 5% CO₂) in an RPMI-1640 media with 10% fetal calf serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Additional supplementation included 1% L-Glutamine and 1% Penicillin-Streptomycin antibiotics (Pen Strep, Thermo Fisher Scientific).
8.4.2 Protein lysis and immunoblot

The cell pellets of each cell line were produced by centrifuging 1 million cells. Next, the cells pellets were treated with a 200-μl radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulphate, and 50 mM Tris-HCl pH 8.0). The RIPA buffer was supplemented with phosphatase and protease inhibitors (Pierce™ 88667 and Pierce™ 88666). The cells were lysed by alternating 3 s on the vortex and 10 min on ice, repeated 3 times. After clearing the cell lysates using centrifugation (16 000 g, 20 min, 4°C), they were loaded onto Criterion TGX precast gels (Bio-Rad). Before loading, cells were boiled for 10 min. Gels were blotted for 45 min (55mA) and then transferred onto nitrocellulose membranes (Trans-Blot Turbo Transfer System, Bio-Rad). A solution of 5% nonfat milk, Tris-buffered saline, and 0.01% Tween-100 was used for blocking and antibody incubations (1 h, room temperature). The membranes were incubated with a rabbit monoclonal anti-MMP14 antibody (Abcam, ab51074, dilution 1:1000) or a rabbit monoclonal anti-PROX1 antibody (Abcam, ab199359, dilution 1:1000), and with a mouse monoclonal anti-beta-actin antibody (Santa Cruz Biotechnology, sc-47778, dilution 1:2500) or a mouse anti-TBG1 antibody (Sigma-Aldrich; T6557). Incubation was carried out at 4°C overnight by gently rocking; before incubating, the secondary antibody the membranes were washed. The secondary antibodies used consisted of an anti-rabbit horseradish peroxidase– (HRP) linked antibody (CST, 7074, dilution 1:2500) and an anti-mouse HRP-linked antibody (CST, 7076, dilution 1:2500). Finally, for chemiluminescent detection, membranes were incubated (1 min at room temperature with gently shuffling) using a 1:1 mix of the WesternBright Sirius HRP substrate components (Advantsta Corporation, K-12043-D20). Bio-Rad’s Chemi Doc XRS+ was used for imaging.

8.4.3 Protein detection by immunofluorescence assay

First, MKN-28, MKN-7, TMK-1, and AGS cells were plated on cover slips in a 24-well plate (10⁵ cells in each well with 500-μl RPMI-1640 media). Fixation of the cells was carried out using a cross-linking reaction with 4% paraformaldehyde in PBS. The cover slips were incubated with the paraformaldehyde solution for 20 min at room temperature. A 0.3% triton-X in PBS solution was used for permeabilization and 1 μg/ml Hoechst for nuclear staining (10 min, room temperature). After washing the cover slips with PBS, blocking was completed by incubation with 0.5% bovine serum albumin (BSA) in PBS (45 min, room temperature). Subsequently, the cover slips were incubated for 1 h in a wet chamber (room temperature) with the primary antibody, rabbit anti-PROX1 (Abcam ab199359, dilution 1:300 in 0.5% BSA in
PBS) or rabbit anti-MMP14 (Abcam ab51074, dilution 1:100 in 0.5% BSA in PBS). The cover slips were washed with PBS and then treated with the secondary antibody; goat anti-rabbit Alexa Fluor 594-conjugated (dilution 1:500 in 0.5% BSA in PBS). Incubation with the secondary antibody was carried out in a wet chamber (1 h, room temperature). Lastly, before mounting the cover slips on the slides with Mowiol 4-88, they were washed with PBS and water. Incubation with the Mowiol 4-88 took place at room temperature overnight. Sigma Panoramic FLASH II (81381) by 3DHistech-Aldrich was used for imaging.

### 8.4.4 Small interfering RNA (siRNA) transfection analysis

The MMP14 and PROX1 protein expressions were studied in AGS cells using small interfering RNA (siRNA) transfection analysis. The cells were plated at a density of 200 000 cells/ml, followed by transfection with either two PROX1 targeting siRNAs (Invitrogen: Stealth RNAi™ HSS 108596; HSS 108597) or with a control siRNA (Invitrogen: 12935200). Transfection was carried out using Thermo Fisher Scientific’s Lipofectamine RNAiMAX, according to the instructions provided by the manufacturer. Two days later, cells were collected and lysed for immunoblot analysis.

### 8.4.5 Quantification of immunoblot band intensities

Quantification of the immunoblot band intensities was carried out using the Fiji colorimetric intensity quantification software (https://imagej.net/Fiji), normalizing the bands to their corresponding loading controls.

### 8.5 Statistical analyses (studies I–IV)

Associations between the biomarker expression levels and the clinicopathological variables were evaluated using the Pearson’s chi-squared test, the Mann–Whitney U-test, or the Kruskal–Wallis test. The Mann–Whitney U-test was also performed to evaluate the significance of the difference in circulating biomarker levels among patients with gastric cancer versus controls. Correlations were evaluated using the Spearman’s rank correlation coefficient. The disease-specific survival for each patient was calculated from the time of surgery until death from gastric cancer. Patients who died from reasons other than gastric cancer were censored at the time of their death. Survival curves were calculated according to the Kaplan–Meier
method and statistical significance was determined using the log-rank test. Hazard ratios (HRs) for uni- and multivariate survival analyses were calculated according to the Cox proportional hazards model applying the enter method. No significant interaction terms were discovered for the biomarkers studied. In the multivariate survival analyses, stage was processed as a categorical covariate. In studies I and IV, optimal cutoff values to dichotomize the circulating biomarker levels into high and low groups were determined using the maximum value of the Youden’s index, except for CRP, where 10 mg/l represented the cutoff value (Youden 1950; Table 10). The receiver operating characteristics (ROC) curve analysis was also used to evaluate the diagnostic properties in addition to calculating the sensitivity and specificity rates by cross tabulation. Across all analyses, we considered a two-tailed p < 0.05 as statistically significant. All statistical analyses were conducted using IBM’s SPSS Statistics for Mac, version 24.0 (IBM Corporation, Armonk, NY, USA).

**Table 10.** Cutoff values for serum MMP14, TAT-2, TATI, the TAT-2/TATI ratio, and plasma CRP.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Cutoff value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP14</td>
<td>0.073 ng/ml</td>
</tr>
<tr>
<td>TAT-2</td>
<td>12.91 ng/ml</td>
</tr>
<tr>
<td>TATI</td>
<td>18.76 ng/ml</td>
</tr>
<tr>
<td>TAT-2/TATI ratio</td>
<td>0.58</td>
</tr>
<tr>
<td>CRP</td>
<td>10 mg/l</td>
</tr>
</tbody>
</table>

Abbreviations: MMP14, matrix metalloproteinase 14; TAT-2, tumor-associated trypsin 2; TATI, tumor-associated trypsin inhibitor; CRP, C-reactive protein.
9 RESULTS

9.1 Distribution of immunoreactivities (studies II and III)

In study II, the roles of PROX1 and MMP14 were examined as potential prognostic biomarkers in gastric cancer, whereas study III investigated TLR1, TLR2, TLR4, TLR5, TLR7, and TLR9 as prognostic biomarkers.

Nuclear PROX1 immunoreactivity was evaluated in 275 cases. PROX1 staining was observed in over 75% of the nuclei in 10 (3.6%) cases, in 50% to 75% of the nuclei in 12 (4.4%) cases, in 25% to 50% of the nuclei in 28 (10.2%) cases, and in less than 25% of the nuclei in 71 (25.8%) cases. Nuclear PROX1 staining was absent in 154 (56.0%) cases.

Table 11 summarizes the distribution of cytoplasmic immunoreactivity for MMP14 and the TLRs.

Table 11. The distribution of cytoplasmic MMP14, TLR1, TLR2, TLR4, TLR5, TLR7, and TLR9 immunoreactivities.

<table>
<thead>
<tr>
<th>Cytoplasmic immunoreactivity</th>
<th>High (%)</th>
<th>Moderate (%)</th>
<th>Low (%)</th>
<th>Absent (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP14</td>
<td>26 (9.4)</td>
<td>54 (19.4)</td>
<td>83 (29.9)</td>
<td>115 (41.4)</td>
<td>278</td>
</tr>
<tr>
<td>TLR1</td>
<td>29 (10.3)</td>
<td>115 (40.8)</td>
<td>120 (42.6)</td>
<td>18 (6.4)</td>
<td>282</td>
</tr>
<tr>
<td>TLR2</td>
<td>44 (16.0)</td>
<td>122 (44.4)</td>
<td>90 (32.7)</td>
<td>19 (6.9)</td>
<td>275</td>
</tr>
<tr>
<td>TLR4</td>
<td>74 (26.3)</td>
<td>106 (37.7)</td>
<td>77 (27.4)</td>
<td>24 (8.5)</td>
<td>281</td>
</tr>
<tr>
<td>TLR5</td>
<td>11 (4.0)</td>
<td>98 (35.4)</td>
<td>114 (41.2)</td>
<td>54 (19.5)</td>
<td>277</td>
</tr>
<tr>
<td>TLR7</td>
<td>30 (11.2)</td>
<td>95 (35.4)</td>
<td>108 (40.3)</td>
<td>35 (13.1)</td>
<td>268</td>
</tr>
<tr>
<td>TLR9</td>
<td>13 (4.7)</td>
<td>130 (46.9)</td>
<td>115 (41.5)</td>
<td>19 (6.9)</td>
<td>277</td>
</tr>
</tbody>
</table>

Abbreviations: MMP14, matrix metalloproteinase 14; TLR, toll-like receptor.

9.2 Association analyses (studies I–IV)

In study I, a high serum MMP14 level associated with stage III through IV cancers as well as with the presence of distant metastases. No other associations were found. Table 12 summarizes the statistical significance levels for the analyses between the biomarkers examined and the clinicopathological variables.

In study II, a high cancer tissue MMP14 expression associated with an older age. We also identified associations between a positive nuclear PROX1 expression and
diffuse-type tumors, and between a positive nuclear PROX1 expression and a high cytoplasmic PROX1 expression level (p < 0.001). We also found a correlation between positive nuclear and high cytoplasmic PROX1 expressions (r_s = 0.310; p < 0.001).

In study III, high TLR2 and TLR4 expressions associated with pT2 through pT4 tumors. A high TLR7 level associated with pT3 tumors, being male, and with stage II disease. Furthermore, an older age associated with a high TLR5 expression and, similarly, with a high TLR9 expression. The expression of each TLR was higher among patients with intestinal-type than among those with diffuse-type tumors. Moreover, TLR expression was coincident, whereby the high expression of each TLR associated with a high expression of other TLRs (all p < 0.001).

**Table 12.** Statistical significance levels for the associations between the studied biomarkers and the clinicopathological variables.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Age</th>
<th>Gender</th>
<th>Stage</th>
<th>T</th>
<th>M</th>
<th>Laurén</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP14</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum^1</td>
<td>ns</td>
<td>ns</td>
<td>0.029</td>
<td>ns</td>
<td>0.022 ns</td>
<td></td>
</tr>
<tr>
<td>Tissue^2</td>
<td>0.041</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>PROX1^2</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>0.041</td>
</tr>
<tr>
<td>TLR1^2</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TLR2^2</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>0.043</td>
<td>ns</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TLR4^2</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>0.049</td>
<td>ns</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TLR5^2</td>
<td>0.028</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TLR7^2</td>
<td>ns</td>
<td>0.021</td>
<td>0.030</td>
<td>0.025</td>
<td>ns</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TLR9^2</td>
<td>0.001</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TAT-2^1</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>0.046</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>TATI^1</td>
<td>0.001</td>
<td>ns</td>
<td>ns</td>
<td>0.030</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>TAT-2/TATI^1</td>
<td>&lt;0.001</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>0.004</td>
</tr>
<tr>
<td>CRP^1</td>
<td>0.024</td>
<td>ns</td>
<td>ns</td>
<td>0.047</td>
<td>ns</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Abbreviations: T, pT classification; M, pM classification; Laurén, Laurén classification; MMP14, matrix metalloproteinase 14; PROX1, prospero homeobox protein 1; TLR, toll-like receptor; TAT-2, tumor-associated trypsin 2; TATI, tumor-associated trypsin inhibitor; CRP, C-reactive protein; ns, not significant.

^1Mann–Whitney U-test.

^2Pearson’s chi-squared test.

In study IV, pT3 through pT4 tumors associated with a high TAT-2, a high TATI, and an elevated CRP level, respectively. Additionally, high TAT-2 and TATI levels associated with each other (p < 0.001). This comparison also revealed a positive correlation (r_s = 0.438; p < 0.001). Furthermore, a high TATI and an elevated CRP level associated with an older age. CRP was elevated among patients with an intestinal-type cancer. Moreover, an association between a high TATI and an
elevated CRP was found (p = 0.037). Finally, a high TAT-2/TATI ratio associated with diffuse-type tumors and with a younger age.

Furthermore, we combined the results from studies III and IV to explore associations between the tissue TLR expression and plasma CRP levels. The median CRP was higher among patients with a high tissue TLR1, TLR4, TLR5, TLR7, or TLR9 expression than among those with a corresponding low TLR expression (unpublished data; Table 13).

**Table 13.** Association between tissue TLR expression and plasma CRP levels among gastric cancer patients (unpublished data).

<table>
<thead>
<tr>
<th>TLR</th>
<th>Median CRP (IQR)</th>
<th>p value¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>2.12 (0.77–7.63)</td>
<td>0.015</td>
</tr>
<tr>
<td>Low</td>
<td>1.12 (0.40–4.18)</td>
<td></td>
</tr>
<tr>
<td>TLR2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>2.14 (0.64–7.47)</td>
<td>ns</td>
</tr>
<tr>
<td>Low</td>
<td>1.33 (0.40–4.39)</td>
<td></td>
</tr>
<tr>
<td>TLR4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>2.19 (0.59–7.76)</td>
<td>0.005</td>
</tr>
<tr>
<td>Low</td>
<td>1.07 (0.38–3.02)</td>
<td></td>
</tr>
<tr>
<td>TLR5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>2.14 (0.75–7.77)</td>
<td>0.029</td>
</tr>
<tr>
<td>Low</td>
<td>1.33 (0.40–4.34)</td>
<td></td>
</tr>
<tr>
<td>TLR7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>2.35 (0.87–9.00)</td>
<td>0.002</td>
</tr>
<tr>
<td>Low</td>
<td>1.17 (0.38–3.88)</td>
<td></td>
</tr>
<tr>
<td>TLR9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>2.75 (0.89–8.88)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Low</td>
<td>0.97 (0.36–3.30)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: TLR, toll-like receptor; CRP, C-reactive protein; IQR, interquartile range; ns, not significant.

¹ Mann–Whitney U-test

9.3 Survival analyses (studies I–IV)

9.3.1 Univariate survival analyses

In study I, 5-year disease-specific survival among patients with a high serum MMP14 was 22.1% (95% confidence interval [CI] 15.2–29.0), compared to 49.2% (95% CI 45.5–52.9; p = 0.001) for those with a low serum MMP14 level (Figure 16).
In the subgroup analyses, a high serum MMP14 level served as an unfavorable prognostic factor among patients with intestinal-type tumors (HR 3.54; 95% CI 1.51–8.33; p = 0.004), those with lymph node metastasis (HR 1.96; 95% CI 1.24–3.09; p = 0.004), those with pT3 through pT4 tumors (HR 1.74; 95% CI 1.13–2.67; p = 0.012), and among men (HR 2.74; 95% CI 1.54–4.87; p = 0.001).

In study II, the 5-year survival among patients with a high tissue MMP14 level was 35.9% (95% CI 24.9–46.9) compared to 45.3% (95% CI 38.0–52.6; p = 0.030) among those with a low tissue MMP14 level (Figure 16).

**Figure 16.** Disease-specific survival for the entire cohort with a low versus a high MMP14 level according to Kaplan–Meier analyses. Calculated according to the log-rank test.

In the subgroup analyses, a high tissue MMP14 level served as an unfavorable prognostic factor among patients with pT3 tumors (HR 1.74; 95% CI 1.04–2.92; p = 0.036), those with lymph node metastasis (HR 1.52; 95% CI 1.06–2.20; p = 0.025), those without distant metastasis (HR 1.51; 95% CI 1.02–2.23; p = 0.042), and, lastly, among those with no nuclear (HR 1.65; 95% CI 1.09–2.51; p = 0.019) or a low cytoplasmic PROX1 staining (HR 1.53; 95% CI 1.07–2.18; p = 0.020).

Nuclear PROX1 expression did not serve as a prognostic factor across the entire patient cohort. However, in the subgroup analyses, a positive nuclear PROX1 expression served as a favorable prognostic factor among men (HR 0.61; 95% CI 0.38–0.98; p = 0.043).

In study III, the 5-year survival among patients with a low tissue TLR5 expression was 37.6% (95% CI 30.0–45.2) compared to 53.4% (95% CI 43.4–63.4; p = 0.014) among those with a high tissue TLR5 expression (Figure 17). The tissue expressions
of TLR1, TLR2, TLR4, TLR7, or TLR9 did not serve as prognostic factors across the entire cohort.

In the subgroup analyses, a high tissue TLR5 expression served as a favorable prognostic factor among patients with intestinal-type tumors (HR 0.58; 95% CI 0.34–0.98; p = 0.043), those without distant metastasis (HR 0.66; 95% CI 0.44–0.99; p = 0.044), those with stage II disease (HR 0.33; 95% CI 0.13–0.83; p = 0.019), and, lastly, among younger patients (HR 0.55; 95% CI 0.32–0.95; p = 0.033). A high tissue TLR7 expression served as a favorable prognostic factor among patients with lymph node metastasis (HR 0.67; 95% CI 0.47–0.96; p = 0.029), those with pT4 tumors (HR 0.51; 95% CI 0.32–0.80, p = 0.003), and among those with stage III disease (HR 0.60; 95% CI 0.38–0.95; p = 0.029). Furthermore, a high tissue TLR9 expression served as a favorable prognostic factor among patients with stage II disease (HR 0.30; 95% CI 0.12–0.76; p = 0.011). TLR1, TLR2, or TLR4 tissue expressions did not serve as prognostic factors for any subgroup.

![Figure 17.](image.png)

**Figure 17.** Disease-specific survival for the entire cohort with low versus high tissue TLR5 expressions based on a Kaplan–Meier analysis. Calculated using the log-rank test.

In study IV, the 5-year survival for patients with a low serum TAT-2 level was 52.2% (95% CI 44.6–59.8) compared to 22.9% (95% CI 11.7–34.1; p < 0.001) among those with a high serum TAT-2 level (Figure 18). The 5-year survival among patients with a low serum TATI level was 52.9% (95% CI 44.7–61.1) compared to 30.6% (95% CI 20.4–40.8; p < 0.001) among those with a high serum TATI level (Figure 18). Both a high serum TAT-2 level and a high serum TATI level served as unfavorable prognostic factors in several subgroups (Table 14).
**Figure 18.** Disease-specific survival for the entire cohort with a low versus high serum TAT-2 and TATI levels based on Kaplan–Meier analyses. Calculated using the log-rank test.

**Table 14.** Survival analysis within subgroups. High serum TAT-2 and TATI levels compared to low levels. Hazard ratios calculated using the Cox proportional hazard model.

<table>
<thead>
<tr>
<th></th>
<th>High serum TAT-2</th>
<th></th>
<th>High serum TATI</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>p value</td>
<td>HR (95% CI)</td>
<td>p value</td>
</tr>
<tr>
<td>Age &lt; 67</td>
<td>ns</td>
<td></td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Age ≥ 67</td>
<td>2.24 (1.35–3.70)</td>
<td>0.002</td>
<td>1.86 (1.15–3.02)</td>
<td>0.012</td>
</tr>
<tr>
<td>Men</td>
<td>1.87 (1.13–3.11)</td>
<td>0.015</td>
<td>1.80 (1.09–2.97)</td>
<td>0.023</td>
</tr>
<tr>
<td>Women</td>
<td>2.12 (1.27–3.55)</td>
<td>0.004</td>
<td>1.87 (1.19–2.96)</td>
<td>0.007</td>
</tr>
<tr>
<td>Stage I</td>
<td>ns</td>
<td></td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>ns</td>
<td></td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Stage III</td>
<td>ns</td>
<td></td>
<td>2.13 (1.33–3.41)</td>
<td>0.002</td>
</tr>
<tr>
<td>Stage IV</td>
<td>2.25 (1.15–4.41)</td>
<td>0.018</td>
<td>2.37 (1.12–4.62)</td>
<td>0.012</td>
</tr>
<tr>
<td>pT1–2 tumors</td>
<td>ns</td>
<td></td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>pT3–4 tumors</td>
<td>1.70 (1.17–2.47)</td>
<td>0.005</td>
<td>1.57 (1.10–2.23)</td>
<td>0.013</td>
</tr>
<tr>
<td>pN0</td>
<td>3.28 (1.27–8.50)</td>
<td>0.014</td>
<td>3.56 (1.38–9.23)</td>
<td>0.009</td>
</tr>
<tr>
<td>pN1–3</td>
<td>1.61 (1.08–2.42)</td>
<td>0.021</td>
<td>1.81 (1.24–2.65)</td>
<td>0.002</td>
</tr>
<tr>
<td>pM0</td>
<td>1.73 (1.12–2.67)</td>
<td>0.014</td>
<td>1.94 (1.31–2.89)</td>
<td>0.001</td>
</tr>
<tr>
<td>pM1</td>
<td>2.25 (1.15–4.41)</td>
<td>0.018</td>
<td>2.37 (1.21–4.62)</td>
<td>0.012</td>
</tr>
<tr>
<td>Intestinal tumors</td>
<td>2.19 (1.05–4.57)</td>
<td>0.037</td>
<td>2.10 (1.10–4.02)</td>
<td>0.025</td>
</tr>
<tr>
<td>Diffuse tumors</td>
<td>1.76 (1.17–2.66)</td>
<td>0.007</td>
<td>1.86 (1.25–2.78)</td>
<td>0.002</td>
</tr>
<tr>
<td>CRP &lt; 10</td>
<td>1.65 (1.10–2.47)</td>
<td>0.015</td>
<td>1.67 (1.14–2.44)</td>
<td>0.008</td>
</tr>
<tr>
<td>CRP ≥ 10</td>
<td>3.67 (1.64–8.22)</td>
<td>0.002</td>
<td>2.51 (1.08–5.84)</td>
<td>0.033</td>
</tr>
<tr>
<td>Low TAT-2</td>
<td>1.80 (1.15–2.83)</td>
<td>0.010</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High TAT2</td>
<td>ns</td>
<td></td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Low TATI</td>
<td>2.30 (1.21–4.36)</td>
<td>0.011</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High TATI</td>
<td>ns</td>
<td></td>
<td>ns</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: TAT-2, tumor-associated trypsin 2; TATI, tumor-associated trypsin inhibitor; CRP, C-reactive protein; HR, hazard ratio; CI, confidence interval; ns, not significant.
Neither CRP nor the TAT-2/TATI ratio served as significant prognostic factors across the entire cohort. However, a high serum TAT-2/TATI ratio predicted a poor prognosis among older patients (HR 2.25; 95% CI 1.39–3.37; p = 0.001).

9.3.2 Multivariate survival analyses

In study I, the serum MMP14 level (HR 1.55; 95% CI 1.02–2.35; p = 0.040), age, stage, and the Laurén classification remained significant prognostic factors in the multivariate survival analysis. In study IV, the serum TATI level (HR 2.01; 95% CI 1.32–3.06; p = 0.001), age, stage, and the Laurén classification remained significant prognostic factors in the multivariate survival analysis.

A combined multivariate survival analysis that included age, stage, Laurén classification, serum and tissue MMP14, tissue TLR5, and serum TAT-2 and TATI levels revealed that among these biomarkers serum TATI emerged as the most important prognostic value (HR 2.09; 95% CI 1.34–3.26; p = 0.001). Stage was processed as a categorical covariate.

9.4 Immunoblot, immunofluorescence, and siRNA transfection analyses (study II)

In study II, we explored the PROX1 and MMP14 expression levels in 11 gastric cancer cell lines by immunoblotting (Figure 19A). Non-simultaneous PROX1 and MMP14 expression was present in OCUM-2MD3, NUGC3, MKN-45, MKN-74, MKN-7, and MKN-28 cells. In OCUM-2MD3 and MKN74, PROX1 was expressed while MMP14 was absent. In NUGC3, MKN-45, MKN-7, MKN-28, and MMP14 all expressed, while PROX1 was absent. Concurrent expression levels were found in OCUM-14, KATOIII, and AGS, although the PROX1 expression signal was much stronger than the MMP14 signal in KATOIII cells. OCUM-14 and TMK-1 exhibited neither the PROX1 nor the MMP14 expression levels in the immunoblot analysis. Actin was expressed uniformly across the blot.

AGS, TMK-1, MKN-7, and MKN-28 were additionally studied by immunofluorescence (Figure 19B). In each cell line, MMP14 expression was recorded on the cell surfaces and in the perinuclear vesicles, whereas the PROX1 expression level was recorded within the nucleus. PROX1 was expressed in all cell lines, although most clearly in AGS. Yet, PROX1 expression was weak in TMK-1,
MKN-28, and MKN-7 cells. The MMP14 expression level was stronger in TMK-1 and MKN-28 cells than in AGS and MKN-7 cells.

Furthermore, PROX1 was silenced in the AGS cells with its effect on MMP14 expression subsequently measured (Figure 19C). A reduction in the PROX1 levels to 0.24- and 0.31-fold was achieved, effectively upregulating the MMP14 levels to 2.01- and 1.85-fold, respectively. These changes in the PROX1 and MMP14 expressions of siRNA-transfected cells were compared to cells treated with a control siRNA. An inverse correlation in the MMP14 and PROX1 expression levels in the immunoblot and immunofluorescence analyses, accompanied by a substantial upregulation of MMP14 levels resulting from PROX1 silencing, shows that PROX1 appears to govern the MMP14 expression level in gastric cancer as well.

**Figure 19.** Immunoblot (A), immunofluorescence (B), and siRNA transfection (C) analyses. Reproduced with permission from Wiley.
9.5 Biomarker levels among patients with gastric cancer versus controls (studies I and IV)

In study I, the serum MMP14 levels were higher among controls than among gastric cancer patients. Among gastric cancer patients, 39 (16.3%) had high and 201 (83.8%) had low MMP14 levels, whereas among controls 14 (29.2%) had high and 34 (70.8%) had low MMP14 levels (p = 0.002).

In study IV, the serum TAT-2 levels were higher among gastric cancer patients (median 8.68 ng/ml; IQR 5.93–13.2) than among controls (median 5.41 ng/ml; IQR 4.12–11.8; p = 0.005; Table 15). The TATI, TAT-2/TATI ratio, or CRP levels did not significantly differ between patients with gastric cancer and controls.

Table 15. Serum TAT-2, TATI, TAT-2/TATI ratio, and plasma CRP levels among gastric cancer patients versus controls. Calculated using the Mann–Whitney U-test.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Gastric cancer</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAT-2 (ng/ml)</td>
<td>5.41 (4.12–11.8)</td>
<td>8.68 (5.93–13.2)</td>
<td>0.005</td>
</tr>
<tr>
<td>TATI (ng/ml)</td>
<td>14.5 (10.3–20.1)</td>
<td>15.9 (11.2–23.5)</td>
<td>ns</td>
</tr>
<tr>
<td>TAT-2/TATI ratio</td>
<td>0.46 (0.26–0.78)</td>
<td>0.55 (0.36–0.86)</td>
<td>ns</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>2.04 (0.42–8.44)</td>
<td>1.62 (0.51–5.81)</td>
<td>ns</td>
</tr>
</tbody>
</table>

Abbreviations: TAT-2, tumor-associated trypsin 2; TATI, tumor-associated trypsin inhibitor; CRP, C-reactive protein; IQR, interquartile range; ns, not significant.
10 DISCUSSION

The declining incidence of gastric cancer results from disentangling its etiological factors and represents a major success story of Western medicine. Treating *H. pylori* infection and improved hygiene have helped prevent a formerly common cancer. Despite this diminishing incidence, prognosis, however, remains poor. More precise subtyping than simple anatomical and histological classifications are necessary in order to advance treatment.

The molecular background of gastric cancer is heterogenous and specific molecular subtypes have been identified. Inflammatory factors clearly play a central role in gastric cancer. In this thesis, several novel inflammation-related biomarkers were studied with the aim of identifying new tools to predict prognosis in gastric cancer.

10.1 Biomarkers

An ideal biomarker meets specific criteria: the biomarker levels are high enough for easy and reliable quantification, the biomarker is involved in tumorigenesis, and changes in the biomarker levels correlate with the disease course. An ideal biomarker also serves as a possible therapeutic target and changes in its levels depict a treatment response. The currently available biomarkers in gastric cancer are useful for monitoring disease progression, but their prognostic value remains low.

Proteases, such as metalloproteinases and trypsin, play a central role in the invasion and metastasis of cancer. Studies I and IV demonstrate for the first time that high serum MMP14 and TAT-2 levels significantly associate with an adverse prognosis in gastric cancer. Study III revealed for the first time that a high tissue TLR5 expression associates with a better prognosis in gastric cancer. Each of these biomarkers prove promising for further investigation, both as means to predict as well as to monitor disease progression and as novel targets for therapy.

10.1.1 MMP14 and PROX1

In studies I and II, both the tissue expression and serum levels of MMP14 were studied with similar results. In each case, a high MMP14 level served as a factor associated with an adverse prognosis. However, a high serum MMP14 level associated with metastasis, not found with tissue MMP14. This may illustrate an
increased hematogenic spread, affecting not only the micro-metastatic gastric cancer cells, but also MMP14 molecules. MMP14 is a membrane-bound protein and its serum-soluble form most likely results from shedding from the membrane. However, an association between serum and tissue MMP14 levels could not be established, indicating that serum MMP14 may also originate from sources other than cancer cells. The results reported in this thesis agree with previous knowledge of MMP14 in cancer related to promoting invasion and metastasis by degrading the ECM (Egeblad and Werb 2002). A high tissue MMP14 expression appeared to serve as an adverse prognostic factor among patients with gastric cancer and associated with advanced disease in several previous studies (He at al. 2013; Peng et al. 2013; de la Peña et al. 2014; Dong et al. 2015; Naseh et al. 2016). For instance, He at al. studied 205 patients, Peng et al. 184 patients, de la Peña et al. 39 patients, Dong et al. 86 patients, and Naseh et al. 96 patients. This thesis examined the largest cohort, consisting of 313 patients with gastric cancer, showing that a high tissue MMP14 expression associates with an unfavorable prognosis.

Most previous studies addressed tissue sections. However, Mimori et al. (2008) showed a high MMP14 gene expression, evaluated using real-time polymerase chain reaction (RT-PCR) from peripheral blood samples, associated with metastasis in a large cohort of 810 patients with gastric cancer. Study I explored the MMP14 protein levels in the sera of 240 patients with gastric cancer with similar results. This indicates that a high serum MMP14 associated with the presence of metastasis and an unfavorable prognosis. In this patient material, the serum MMP14 levels were higher among controls than among patients with gastric cancer. Previously, the MMP14 tissue expression level was elevated in gastric cancer tissue sections compared to healthy gastric mucosa (de la Peña et al. 2014; Dong et al. 2015; Naseh et al. 2016). However, the results from serum and tissue levels are not always directly comparable. Additionally, the regulatory mechanisms behind MMP14 shedding remain largely unknown. MMP14 is not specific to malignant disease and its presence in the serum of patients with benign disease may result from reasons other than cancer.

Li et al. (2019b) recently showed that silencing MMP14 in gastric cancer cells using short hairpin RNAs (shRNA) inhibited invasion. In subgroup analyses, we showed here that both high serum and high tissue MMP14 expressions served as prognostic factors among patients with gastric cancer with locally advanced disease. These results along with Li et al.’s demonstrate the relevance of MMP14 in the local spread of a tumor.
A high tissue MMP14 expression predicted an unfavorable prognosis particularly among patients with a low PROX1 immunoreactivity — either no nuclear PROX1 reactivity or a low cytoplasmic reactivity — suggesting a connection between MMP14 and PROX1 in gastric cancer. Gramolelli et al. (2018) recently demonstrated that PROX1 suppresses the transcription of MMP14 in multiple cancers. That study did not, however, include gastric cancer. In study II, the expression of MMP14 and PROX1 was investigated in 11 gastric cancer cell lines, revealing an inverse correlation between the levels of MMP14 and PROX1 in seven cell lines. Additionally, the silencing of PROX1 resulted in increased MMP14 levels. These results suggest that PROX1 also contributes to the regulation of MMP14 expression in gastric cancer. However, an association between tissue MMP14 and PROX1 expression levels was not found among the patients with gastric cancer we studied, demonstrating that MMP14 regulation in vivo in gastric cancer also includes factors other than PROX1.

PROX1 is a controversial biomarker in gastric cancer. It has previously been paradoxically reported to associate, on the one hand, with a favorable, and on the other hand, with an unfavorable prognosis among patients with gastric cancer. For instance, Laitinen et al. (2017) showed a high cytoplasmic tissue expression of PROX1 associated with a better prognosis among the same patient cohort studied in this thesis. In comparison, Ueta et al. (2018) found that a high nuclear PROX1 expression associated with an adverse prognosis among 99 surgically treated patients with gastric cancer. According to current knowledge, PROX1 functions as a transcription factor and, thus, analyzing nuclear expression levels represents the most relevant means of quantification (Elsir et al. 2012). Study II aimed to clarify the discrepancy of the results by evaluating the prognostic qualities of nuclear PROX1 immunoexpression in this patient cohort. No association between prognosis and nuclear PROX1 expression could be established. However, a positive nuclear PROX1 expression associated with diffuse-type gastric cancer, known to feature a poor prognosis. None of the patients studied by Ueta et al. received preoperative chemo- or radiotherapy. Unfortunately, the corresponding information was not available here. Moreover, Ueta et al. compared tissues with over 50% PROX1-positive nuclei to tissues with less than 50% PROX1-positive nuclei, whereas we compared tissues with PROX1-positive nuclei to tissues with PROX1-negative nuclei.

The role of cytoplasmic PROX1 remains unknown. In the context of transcription factors, cytoplasmic immunoreactivity may reflect dysfunctional nuclear transport. Correspondingly, a high cytoplasmic PROX1 immunoreactivity may result from a
dysfunction in the transport of PROX1 to the nucleus and, hence, high cytoplasmic levels may actually reflect low nuclear levels. However, no such association was discovered here. Instead, an association was controversially discovered between positive nuclear and high cytoplasmic PROX1 expressions. Thus, further studies are needed to clarify the role of PROX1 as a prognostic biomarker in gastric cancer.

10.1.2 Toll-like receptors (TLRs)

In study III, we showed that TLR5 tissue expression served as a favorable prognostic factor. In the subgroup analyses, a high TLR5 level served as a marker of a favorable prognosis particularly among younger patients, patients with stage II disease, those with no metastasis, and among those with intestinal-type cancer. Interestingly, TLR5 activation was previously shown to facilitate tumor growth in vitro (Park et al. 2011). In that study, TLR5 activation by flagellin promoted the proliferation of gastric cancer cells. While in vitro studies are convenient for exploring specific signaling pathways, they are not often directly comparable to an in vivo setting given the lack of a three-dimensional tumor microenvironment and the plethora of coexisting signaling molecules.

A high TLR3 expression, evaluated using immunohistochemistry, was previously shown to associate with an adverse outcome (Fernandez-Garcia et al. 2014). Unfortunately, TLR3 levels were not analyzed in this study. Yang et al. (2014) concluded that a high TLR2 expression, evaluated using immunohistochemistry, associated with the presence of metastasis. We did not observe such an association in our series, although we found an association between a high TLR2 expression and locally advanced tumors. This likely stems from the differences in study designs, since we evaluated the staining intensity of the cancer cells and grouped the data into low and high expression groups. Yang et al., however, also considered the percentage of stained cancer cells resulting in a combined expression score. Yet, they included 47 patients with gastric cancer, whereas our cohort consisted of 313 individuals.

Some researchers suggest that TLR9 signaling is relevant in *H. pylori*–induced gastric carcinogenesis (Qin et al. 2019). In that study, the co-cultivation of gastric cancer cells with *H. pylori* accelerated growth via the increased TLR9 activation. By contrast, we found that a high TLR9 expression in tissue samples associated with a better prognosis among stage II patients. The clinical data from study III did not include information on patients’ *H. pylori* infection status, and, thus, comparisons between the TLR expression levels and the presence of *H. pylori* were impossible.
This is unfortunate, since *H. pylori* induces extensive TLR expression in the gastric mucosa and several TLR single-nucleotide polymorphisms contribute to gastric carcinogenesis by affecting the crosstalk between our immune system and *H. pylori* (Smith 2014; Pachathundikandi et al. 2015; Ravishankar et al. 2015; Xu et al. 2017). Among all TLRs analyzed, we identified associations between intestinal-type tumors and the high expression of each TLR examined. Furthermore, the high expression of each TLR significantly associated with a high expression of other TLRs. This suggests that local inflammation of the gastric mucosa, characterized by a polymorphous TLR expression, is particularly important in the context of intestinal-type gastric cancer.

Data were combined from studies III and IV to explore the associations between tissue TLR expression and plasma CRP levels, revealing that a high CRP associates with high TLR1, TLR4, TLR5, TLR7, and TLR9 tissue expressions in gastric cancer. Inflammation within the gastric mucosa and the resulting TLR signaling activation may result in high concentrations of local interleukins and TNF-α, possibly further transferring to systemic circulation and stimulating CRP secretion from hepatocytes (Caruso et al. 2007).

### 10.1.3 TAT-2, TATI, and CRP

In study IV, we explored the preoperative serum TAT-2 and TATI and plasma CRP levels as biomarkers among patients with gastric cancer. Serum TAT-2 and TATI served as prognostic factors, with the serum TATI remaining an independent prognostic factor in the multivariate survival analysis. Serum TAT-2 has not been previously studied in relation to survival in gastric cancer. Here, we showed for the first time that a high serum TAT-2 expression may identify patients with gastric cancer likely to experience an unfavorable prognosis. Furthermore, the TAT-2 levels were higher among patients with gastric cancer than among controls.

TATI is a multifunctional protein, which can promote carcinogenesis through its growth factor capabilities while also suppressing invasiveness through inhibiting trypsin (Ozaki et al. 2009; Itkonen and Stenman 2014). This phenomenon is also seen in prognostic studies. Similar to this study, a high preoperative serum TATI level was previously found to associate with an advanced cancer stage and the presence of metastasis (Kemik et al. 2013). Kemik et al. determined the serum TATI levels in 90 patients with gastric cancer. This study validates serum TATI as an adverse prognostic biomarker in 313 patients with gastric cancer. In both studies, TR-IFMA was used to determine the serum TATI levels. Given the tumor-
suppressive capabilities of TATI, its expression in gastric cancer tissues was previously described as associating with a low cancer stage and a better prognosis (Wiksten et al. 2005). Additionally, TATI appears to modulate apoptosis in certain cancers (including hepatocellular, breast, and colorectal cancers), further complicating its signaling in carcinogenesis (Räsänen et al. 2016). Thus, it is unsurprising that studies of TATI in cancer have yielded discordant results.

The prognostic values of serum and tissue MMP14, tissue TLR5, and serum TAT-2 and TATI were compared in a separate multivariate analysis. That analysis revealed that serum TATI carried the strongest prognostic value in this system. The hazard ratio for a high serum TATI (2.09) nearly reached that for diffuse-type tumors (2.14).

TATI has also been shown to function as an acute-phase reactant, where its levels resemble that of other acute-phase reactants, including CRP and ferritin (Ogawa 1988; Solakidi et al. 2004). In this study, a high TATI associated with an elevated CRP. High TATI levels are often accompanied by high levels of tumor-associated trypsin (Stenman et al. 1991; Itkonen and Stenman 2014). We also found a correlation between high serum TAT-2 and TATI levels.

Among patients with gastric cancer, an elevated preoperative CRP level was previously found to serve both as a diagnostic and as a prognostic biomarker and to associate with deeper tumors, the presence of metastasis, and a higher risk of recurrence (Chang et al. 2010; Nozoe et al. 2011; Yu et al. 2013). In this study, we could not establish an association between the CRP level and prognosis, nor did we detect differences among the CRP levels between patients with gastric cancer and controls. However, similar to previous studies, study IV showed that an elevated CRP associated with locally advanced disease. Large primary tumors appear to secrete ample amounts of inflammatory mediators into systemic circulation stimulating CRP secretion in the liver (Caruso et al. 2007).

A meta-analysis by Yu et al. (2013), consisting of 2597 patients, examined 12 original studies, 9 of which found that CRP served as a prognostic factor among patients with gastric cancer. Similar to this study, three of those studies failed to show a significant connection between the CRP level and prognosis. It remains unclear why studies on similar patient cohorts with similar methodologies continue to yield discordant results. One possible explanation may be that the etiology of an elevated preoperative CRP in gastric cancer is not always comparable. A malignant tumor may cause not only local but also systemic inflammation in the body, resulting
in elevated CRP levels (Caruso et al. 2007). Alternatively, inflammation of the gastric mucosa itself, such as due to \textit{H. pylori} infection, may elevate the CRP level while also increasing the risk of gastric cancer (Francescone et al. 2015).

\section*{10.2. Strengths and limitations of the study}

The study material within this thesis consists of a TMA series of 313 surgically treated patients with gastric cancer at Helsinki University Hospital between 2000 and 2009. The strengths of these studies include the large patient cohort with reliable and precise clinical data and frequently updated follow-up information. Other strengths include the reliable laboratory methodology routinely validated through several previous studies, either in the context of gastric cancer or other cancers of the gastrointestinal tract.

The retrospective study design did not allow us to retrieve consecutive data on all known prognostic and risk factors in gastric cancer. These clinicopathological characteristics include, for example, lymphatic emboli, the tumor subsite, and venous and neural invasion. Additionally, information regarding the \textit{H. pylori} infection status was unavailable to us.

Given the use of several parallel patient record software programs at Helsinki University Hospital, it was impossible to obtain precise information regarding the pre- and postoperative chemotherapy administered. We also recognize that some patients interrupted their adjuvant therapy early during treatment because of adverse events. In these cases, it is difficult to definitively state whether they should be considered as belonging to the chemotherapy group or not. The inclusion of incomplete data weakens the scientific value of this study, and, thus, we did not include administered chemotherapy in our analyses. This remains a confounding factor.

In the subgroup analyses, we decided not to adjust the p values, and instead present exploratory overviews for the study cohort with raw p values. Each method which adjusts p values or the significance threshold introduces its own inaccuracies. Thus, presenting raw p values serves as an indisputable means of analysis and we chose to leave the criticism regarding multiple tests to readers. Nevertheless, it is true that while conducting multiple subgroup analyses the probability for family-wise errors increases.
Finally, we acknowledge that while our study provides novel and interesting findings on prognostic biomarkers in gastric cancer, further studies are necessary to corroborate our findings and to test their utility.

10.3 Clinical applications and future prospects

Currently, none of the biomarkers studied in this thesis are in routine clinical use in gastric cancer, although they show potential for further investigations. Adjuvant therapy is always needed along with surgery to eradicate micrometastases. Chemotherapy and radiotherapy are capable of improving prognosis; however, their effectiveness varies between patients. Individualized medicine stratifies patients into groups providing specific treatment to patients with specific qualities. The only individualized therapy currently in clinical use in gastric cancer is the administration of the HER2 antagonizing antibody — trastuzumab — to HER2-positive patients. The further individualization of treatment in gastric cancer remains crucial to improving patient prognosis in the future.

The results from this thesis on MMP14, accompanied by the results from previous studies, show that MMP14 represents a promising prognostic biomarker and a target for therapy in gastric cancer. MMP inhibitors have long fallen within the scope of cancer-drug development (Brown 1997; Winer et al. 2018). MMPs are a logical target molecule for specific therapy, since they play important functions in invasion and metastasis. Antagonizing them aims to prevent or diminish the spread of cancer cells. Today, focus has switched from broad-spectrum MMP inhibitors to selective inhibitors with the aim of improving efficacy and reducing side effects (Jacobsen et al. 2010). Currently, no inhibitor for MMP14 is available for clinical use. However, studies evaluating MMP14 as a target molecule for specific therapy remain ongoing with promising preliminary results. Arkadash et al. (2018) recently established a methodology to generate modified TIMP2 with a greatly improved specificity to MMP14.

TLRs have been proved promising targets for therapy in several cancers, particularly in combination with radio- and chemotherapy (Mikulandra et al. 2017). Both agonism and antagonism carry therapeutic potential depending upon the disease. However, overly stimulating the TLR pathways may also result in severe side effects, such as inducing autoimmune responses. Balance is key to regulating the TLR pathways. In the context of gastric cancer, TLR7 agonism by imiquimod was shown to inhibit the growth of gastric cancer cells in vitro (Jiang et al. 2016).
Treating gastric cancer cells using imiquimod may directly trigger programmed cell death while also activating the dendritic cells and stimulating them to secrete type I IFN. Currently, imiquimod is used to treat certain skin conditions, including small basal cell carcinomas and solar keratosis.

In studies II and III, the immunoreactivity of tissue specimens was visually quantified by a team of two independent researchers. Automated digital scoring systems are quickly developing and appear helpful in analyzing large patient cohorts and evaluating the visual density with the aim of mimicking the expertise of a trained pathologist (Rizzardi et al. 2012). Some automated systems are already capable of identifying individual cells. However, they cannot accurately analyze cell morphology and single out malignant cells from the surrounding non-malignant stroma (Choudhury et al. 2010; Akbar et al. 2015; Meyerholz and Beck 2018). Reliably identifying cancer cells from the stroma is essential in order to produce accurate and reproducible data. Additionally, within the context of inflammatory biomarkers, immune cell habitation of the tumor microenvironment and their polymorphous protein expression limits the use of automated systems. Digital colorimetric quantification is not yet capable of replacing visual evaluation; however, the automated systems appear extremely promising and are expected to become routinely available in the future.
11 CONCLUSIONS

I A high serum MMP14 level in gastric cancer may serve as a marker of an unfavorable prognosis.

II A high MMP14 tissue expression may serve as a prognostic factor associated with an unfavorable prognosis. Prognosis seems particularly poor among those with high MMP14 and low PROX1 expression levels in the tumor tissue. PROX1 appears to affect MMP14 expression in gastric cancer.

III A high TLR5 tissue expression level may indicate a better prognosis among patients with gastric cancer, particularly among those with intestinal-type tumors or stage II disease. TLR1, TLR2, TLR4, TLR7, and TLR9 do not serve as prognostic factors despite their high expression levels in intestinal-type tumors.

IV High serum TAT-2 and TATI levels may serve as markers of a poor prognosis among patients with gastric cancer. The TAT-2/TATI ratio or preoperative plasma CRP did not serve as prognostic factors.
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Many of my friends are also aspiring researchers themselves and I am grateful to have had the opportunity to share this journey towards the doctoral degree with them. After a busy day of science relaxing with my friends has always left my mind (more or less) refreshed. I am truly grateful for such friends and without them my studies would not have been the same.

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