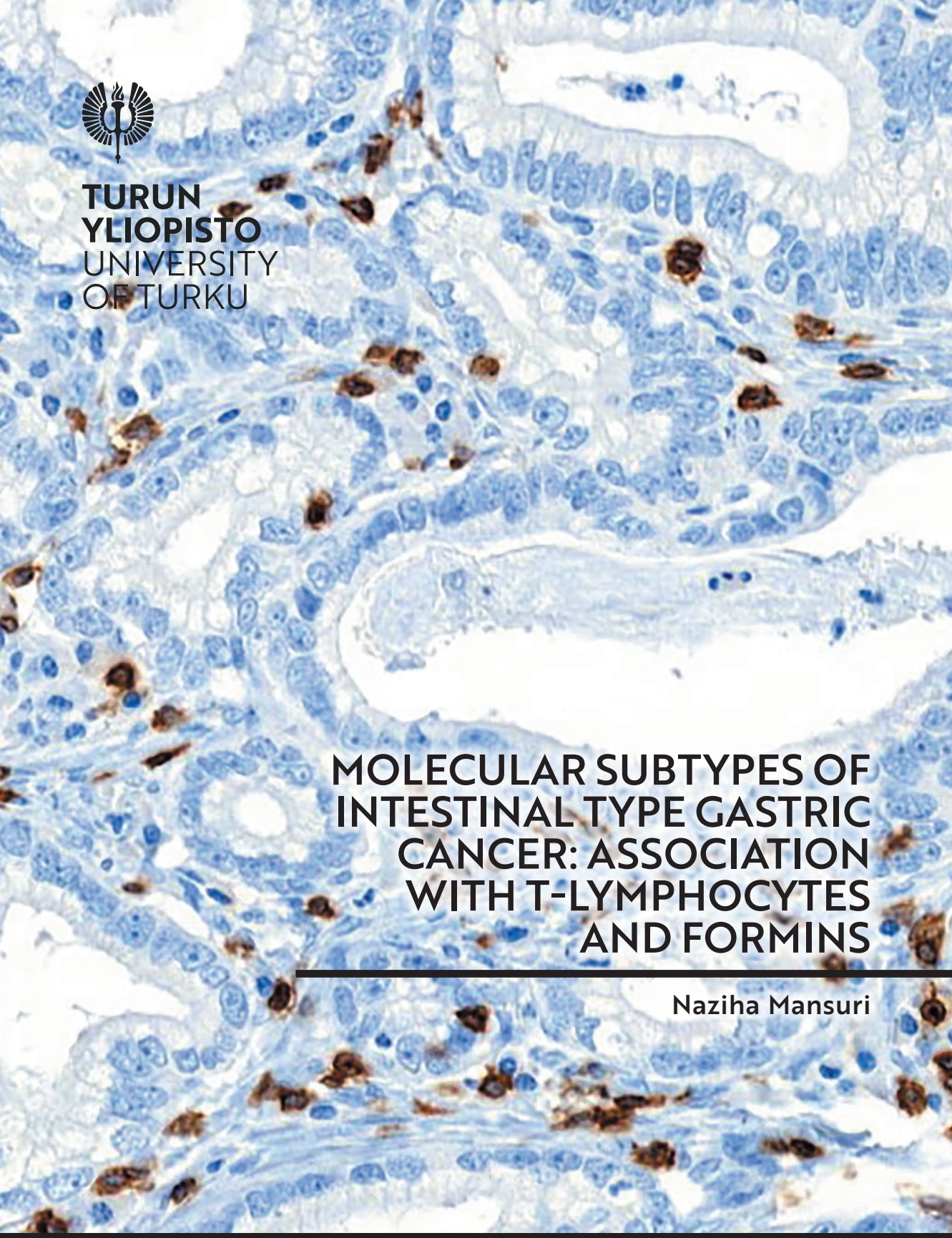

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“I never lose. I either win or learn.”
Nelson Mandela

To Mansur, my soul mate and my ultimate supporter,
To Rasha, Rami, and Ramez, the true meaning of my life,
To my mother, my father, and my father-in-law, may their soul rest in peace.

UNIVERSITY OF TURKU
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ABSTRACT

Despite the decline of gastric cancer (GC) incidence in Western countries over the last decade, it is still one of the most significant causes of cancer mortality worldwide. The traditional morphology-based grading systems, including the world health organization (WHO) and Lauren's grading systems, have limited applicability in managing treatment choices, as they poorly catch the molecular heterogeneity of GC. Thus, classifications based on molecular features are needed. Recent genome analyses have shown that GC consists of several molecular subtypes characterized by distinct alterations. In our study, we used tissue-based methods, *i.e.*, immunohistochemistry and in situ hybridization, in the molecular classification of GC, emphasizing the intestinal subtype. Our results show that GC can be divided into four non-overlapping subtypes based on *Epstein-Barr virus* (EBV) positivity, mismatch repair protein (MMR), and TP53 aberration status. In conclusion, GC molecular subtyping can be performed with a simple methodology applicable to clinical routine.

Host immune response is an important predictive and prognostic factor in many cancer types, including GC. Detailed information on the accumulation of tumor-infiltrating T lymphocytes in the different molecular GC subtypes and their prognostic correlation is scarce. We analyzed the presence of CD3+, CD8+, and FOXP3+ (Forkhead box P3) T lymphocytes in the molecular subtypes of intestinal-type GC. We found that EBV+ cancers harbor increased lymphocyte infiltration and a high CD8+/FOXP3+ ratio. In addition, we found that high numbers of CD8+ and CD3+ T lymphocytes are associated with better survival, and their accumulation is an independent prognostic factor.

Formin proteins regulate the actin cytoskeleton and cell migration and play an essential role in cancer call functions. However, the expression and clinical association of formins in GC remains largely undiscovered. Here we analyzed the expression of FHOD1 and FMNL1 formins in GC cell lines and clinical samples of intestinal-type GC. We found that FHOD1 expression in cancer cells correlated with high intratumoral CD8+ T lymphocyte infiltration. Reduced FHOD1 expression was seen in the tumors with aberrant TP53. FMNL1 expression in cancer cells was associated with the size of the tumors and the stage of the disease. The results demonstrate a link between FHOD1 and FMNL1 expression with biological features of GC. However, we did not find a correlation between formin expression and GC prognosis.

KEYWORDS: Gastric cancer, tissue microarray, IHC, molecular classification, formin, tumor-infiltrating lymphocyte.

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Lääketieteellinen tiedekunta
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TIIVISTELMÄ

Vaikka mahasyövän esiintyminen on viime vuosikymmeninä laskenut kehittyneissä maissa, se on edelleen yksi tärkeimmistä syöpäkuolemien aiheuttajista. Mahasyöpä on biologialtaan monimuotoinen. Tästä syystä perinteinen kudosmorfologiaan perustuva luokittelu, kuten WHO:n tai Laurénin luokitus, hyödyttää hoitopäätöksien tekoa vain rajallisesti. Molekulaarisiin piirteisiin perustuvan luokituksen kehittäminen olisikin tärkeää. Genomianalyyseihin perustuva tutkimus on osoittanut, että mahasyöpä koostuu useista molekulaarisista alatyypeistä. Analyysimenetelmät ovat kuitenkin monimutkaisia ja vaativat erityisosaamista. Tutkimuksessamme käytimme kliiniseen käyttöön soveltuvia menetelmiä; immunohistokemiaa ja in situ-hybridisaatiota mahasyöpien luokitteluun. Tulosten perusteella yksinkertaisen algoritmin avulla mahasyövät voidaan luokitella eri alaryhmiin *Ebstain-Barr virus*-positiivisuuden, TP53 poikkeavuuden ja MMR-puutoksen perusteella. Kliinisesti eroaviin alaryhmiin tapahtuva jaottelu voidaan siis toteuttaa yksinkertaisella ja kliiniseen diagnostiikkaan soveltuvalla menetelmällä

Immuunijärjestelmän toiminta on tärkeä syövän ennusteen kannalta. Yksityiskohtaista tietoa siitä, miten T solut hakeutuvat eri mahasyövän alatyyppeihin ja liittyvät taudin käyttäytymiseen ei toistaiseksi ole. Tässä tutkimuksessa analysoimme CD3, CD8 ja FOXP3 antigeenejä ilmentävien T-lymfosyyttien esiintymistä intestinaalisen mahasyövän alatyypeissä. Tulosten perusteella EBV+ syövissä on runsaimmin T-lymfosyyttejä ja korkein CD8+/FOXP3-suhde. Totesimme myös, että runsas T-lymfosyyttien määrä korreloi intestinaalisen mahasyövän parempaan ennusteeseen ja toimii itsenäisenä ennustetekijänä.

Formiiniproteiinit säätelevät solujen aktiinitukirankaa ja solujen migraatiota, ja ovat tärkeitä syöpäsolujen toiminnassa. Kuitenkin formiinien esiintymistä ja tehtäviä mahasyövässä tunnetaan huonosti. Tässä tutkimuksessa analysoimme FHOD1 ja FMNL1 formiineja mahasyöpäsolulinjoissa ja intestinaalista alatyyppiä edustavien mahasyöpien kudosnäytteissä. Kasvainsolujen FHOD1:n ilmentyminen oli yhteydessä korkeaan intratumoraalisten T-lymfosyyttien määrään. FHOD1:n alentunutta ekspressiota nähtiin syövän alatyypissä, johon liittyy TP53 mutaatio. FMNL1:n ilmentyminen puolestaan korreloi kasvaimen kokoon ja taudin leviämisasteeseen. Tulosten perusteella FHOD1 ja FMNL1-formiinien ilmentyminen liittyy mahasyövän biologisiin piirteisiin, joskaan ilmentymisellä ei näytä olevan yhteyttä mahasyövän ennusteeseen.

AVAINSANAT: Mahasyöpä; IHC; molekyyliluokitus; formiini; kasvaimeen tunkeutuva lymfosyytti.

Table of Contents

Abbreviations

ACRG	Asian Cancer Research Group
ADP	adenosine diphosphate
APCs	Antigen-presenting cells
Arp2/3	actin-related protein 2/3
BARF1	BamHI-A rightward frame 1
BARTs	BamHI-A rightward transcripts
CALs	Cancer-associated lymphocytes
CD	Cluster of differentiation
CDH1	cadherin1/ E-cadherin, epithelia cadherin
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CPG	cytosine guanine dinucleotide
DAB	3,3'-diaminobenzidine
DNA	deoxyribonucleic acid
DRF	Diaphanous-related formin
EBER	EBV-encoded small RNA
EBNA1	EBV nuclear antigen 1
EBV	Epstein-Barr virus
EMT	epithelial-mesenchymal transition
FAP	familial adenomatous polyposis
FFPE	formalin-fixed paraffin-embedded
FHOD1	Formin-homology domain-containing protein 1
FMNL1	Formin-like protein 1
F-actin	filamentous actin
FOXP3	The transcription factor forkhead box P3
G-actin	globular actin
GFP green	fluorescent protein
GTPase	guanosine triphosphatase
GAPPS	Gastric adenocarcinoma and proximal polyposis of the stomach
GOJ	gastro-oesophageal junction
GS	Genomic stability

H&E	haematoxylin-eosin
HER2	erbB-2/human epidermal growth factor
HNSCC	Head and neck squamous cell carcinoma
IFN-γ	interferon-γ
IHC	immunohistochemistry
LOH	loss of heterozygosity
MAPK	Mitogen-activated protein kinase pathways
MHC1	Major histocompatibility class I molecules
MLH1	MutL homolog 1/ DNA mismatch repair protein
MMR	mismatch repair
MMR-D	mismatch repair protein deficient
MMR-P	mismatch repair protein proficient
miRNA	micro ribonucleic acid
mRNA	messenger ribonucleic acid
MSH2	DNA mismatch repair protein MSH2/3/6
MSH6	MutS homolog 6
MSI	microsatellite instability
MSI-H	microsatellite instability-high
MSI-L	microsatellite instability -low
MSS	microsatellite-stable
mTOR	mechanistic target of rapamycin
ngTMA	next-generation tissue microarray
PD-L1/2	programmed cell death ligand 1/2
PMS2	mismatch repair endonuclease PMS2
PI3K–AKT–mTOR	phosphatidylinositol 3-kinase–AKT mechanistic target of rapamycin
RFS	recurrence-free survival
SRF	serum response factor
T regs	T regulatory cells
TCGA	The Cancer Genome Atlas
TNM	TNM classification of malignant tumors (tumor, node, metastasis)
TP53	cellular tumor antigen p53
VEGF	vascular endothelial growth factor
WHO	World Health Organization
wt	wild-type

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their roman numerals:

I Birkman, E. M., Mansuri, N., Kurki, S., Ålgars, A., Lintunen, M., Ristamäki, R., Sundström, J., & Carpén, O. (2018). Gastric cancer: immunohistochemical classification of molecular subtypes and their association with clinicopathological characteristics. *Virchows Archiv: an international journal of pathology*, *472*(3), 369–382. *, **

II Mansuri, N., Birkman, E. M., Heuser, V. D., Lintunen, M., Ålgars, A., Sundström, J., Ristamäki, R., Lehtinen, L., & Carpén, O. (2021). Association of tumor-infiltrating T lymphocytes with intestinal-type gastric cancer molecular subtypes and outcome. *Virchows Archiv: an international journal of pathology*, *478*(4), 707–717.

III Mansuri N, Heuser V D, Birkman E-M, Ålgars A, Sundström J, Ristamäki R, Carpén O, Lehtinen L. (2021). FHOD1 and FMNL1 formin proteins in intestinal gastric cancer: correlation with tumor-infiltrating T lymphocytes and molecular subtypes. *Gastric Cancer* 24**,** 1254–1263.

* This article was included in the thesis of Eva-Maria Birkman (ISSN 2343-3213)
**Eva-Maria Birkman and Naziha Mansuri have an equal contribution.

1 Introduction

Gastric cancer (GC) is a common malignancy and a common cause of cancer-related deaths worldwide (Ferlay et al., 2019; Arnold et al., 2020). Adenocarcinoma is the most common type of gastric cancer, and by Laurén classification, is divided into two distinct morphological types; intestinal and diffuse (Laurén, 1965). Lauren's traditional histological classification of gastric adenocarcinoma is still the most practical tool in diagnosing GC. Intestinal-type GC is more often sporadic and linked to environmental factors than diffuse-type GC, which is highly metastatic and characterized by rapid disease progression and a poor prognosis (Laurén, 1965).

Recently, a large study provided by the Cancer Genome Atlas Association (TCGA) has classified gastric cancer into four molecular subtypes based on genomic alterations (Bass et al., 2014); these subtypes are characterized by either *Epstein-Barr virus* (EBV) infection, microsatellite instability (MSI), chromosomal instability (CIN), or genomic stability (GS). The TCGA and similar studies have provided important information about the heterogeneity of gastroesophageal cancer; however, the complex methodologies used in these studies are not readily applicable for routine clinical diagnostics (Sohn et al., 2017). In our study, using a tissue microarray (TMA) from a cohort of 244 adenocarcinomas of the stomach, the gastroesophageal junction (GOJ), and distal esophagus, we have been able to identify four non-overlapping subgroups of GC tumors by combining the Laurén classification, mismatch repair (MMR) protein and TP53 immunohistochemistry (IHC) and EBER in situ hybridization (ISH).

The tumor microenvironment (TME), consisting of surrounding blood vessels, immune cells, fibroblasts, and the extracellular matrix (ECM), is implicated in tumorigenesis. The interaction between the different components of TME, including the crosstalk between tumor cells and the surrounding cancer-associated lymphocytes (CALs), influences the development and progression of cancer. Understanding the mechanism of interactions between different TME components and cancer cells provides critical information in the context of targeted therapies. Cancer-associated lymphocytes, including cytotoxic CD8+ T lymphocytes, are crucial for tumor surveillance. They can kill tumor cells, while T-regulatory cells, mainly FOXP3 + cells,

contradict the action of CD8+ T lymphocytes. The immunosuppressive balance between CD8+ cytotoxic cells and the T-regulatory cells regulates tumor progression (Yu et al., 2018). The type and amount of intratumoral T lymphocytes may indicate the progression in many cancer types, including colon, lung, and melanoma (Shang et al., 2015). However, the exact role of immune cells in GC is less clear, especially in association with the different molecular subtypes of intestinal gastric cancer.

Invasive growth by cancer cells requires motility regulated by the actin cytoskeleton. Therefore, proteins that guide actin-polymerization can affect cancer progression. Among actin-organizing proteins are formins, a family consisting of fifteen members in mammals (Goode & Eck, 2007). Among them, FHOD1 is linked to epithelial-to-mesothelial transition, an essential feature for cancer cell migration and metastasis (Heuser et al., 2018), while FMNL1 is connected to lymphocyte activity and hematological malignancies (Thompson et al., 2020). The exact prognostic role of FHOD1 and FMNL1 in intestinal gastric cancer remains uncovered.

This thesis studied whether tissue-based markers can be surrogates for intestinal-type gastric cancer molecular subtyping. We also examined the intratumoral immune infiltrates, especially CD8+, CD3+, and FOXP3+ T lymphocytes, concerning molecular subtypes and outcome and the expression of FHOD1 and FMNL1 formins in clinical intestinal GC tumor samples and GC cell lines.

2 Review of the Literature

2.1 Epidemiology of Gastric cancer

Gastric cancer (GC) has been reported in the hieroglyphic script from ancient Egypt. According to Verona's statistical analysis of cancer epidemiology in Italy from 1760–1839, the first statistical analysis of GC incidence and mortality was the most common cancer with high mortality (Carlotto et al., 2019). Less than ten years ago, it was the most prevalent cancer globally (Sonnenberg & Baron, 2010; Balakrishnan et al., 2017). Although absolute numbers and the age-standardized rates of stomach cancer have declined, in 2012, GC was the fifth most common malignancy with an estimated 952 000 new cases worldwide (Rawla & Barsouk, 2019). In 2018, and based on GLOBOCAN data, GC remains the fifth most common neoplasm and the third most lethal cancer worldwide (Bray, 2018). GC is the fourth most common cancer in men and seventh most common cancer in women (Ferlay et al., 2019; Fitzmaurice et al., 2019).

In Finland, and according to the Finnish Cancer Registry, the number of new gastric cancer cases has decreased considerably in recent decades. In 2019, gastric cancer was more common in men than women; age-adjusted incidence was 13.9/100 000 for men and 7.2/100 000 for women. The age-standardized 5-year survival of gastric cancer was 26% for men and 30% for women.

Histologically, 90% of gastric cancer are adenocarcinomas. Additional rare malignancies include gastric lymphoma, mainly the mucosa-associated T cell lymphoma (MALT) type, gastrointestinal stromal tumors (GISTs), neuroendocrine tumors (NETs), and other lesions with undetermined malignant potential (Nagtegaal et al., 2020). This thesis concentrates on gastric adenocarcinoma, mainly the intestinal type.

GC has heterogeneous geographic distribution, and its incidence varies significantly between high-risk and low-risk countries (Forman & Burley, 2006). The geographic variability correlates with the rate of *Helicobacter pylori (H. pylori)* infection rates. Globally, East Asia, particularly China and Korea, contributes to the high incidence rate of gastric cancer. At the same time, the US and Europe show a low incidence of *H. pylori* and consequently have a low incidence of gastric cancer (Kim et al., 2017). The age-standardized incidence and death rates have declined steadily also in high-risk countries, which could reflect the eradication of *H. pylori* infection in these areas (Lee et al., 2016). Over the last decades, there has been an improvement in the

survival and incidence of non-cardiac gastric adenocarcinomas (non-Cardia GC often associated with *H. pylori* infection), while the cardiac adenocarcinomas remain unchanged (Asplund et al., 2018). However, despite the decline in the incidence rate of GC, the prognosis of gastric cancer remains poor, especially in the advanced disease stage. Unquestionably, there is an increasing need to identify new biomarkers that can help improve the individual patient's prognosis and enhance the best treatment options.

2.2 Etiology and molecular risk factors

Gastric cancer is multifactorial. Generally, non-cardiac gastric cancer is facilitated by chronic inflammation and environmental factors. In contrast, the pathogenesis of cardiac gastric cancer remains less clear. Other risk factors linked with non-cardiac gastric cancer are smoking, alcohol intake, high salt in the diet, and obesity (Rawla & Barsouk, 2019). Two etiological factors have been thoroughly investigated and shown to be associated with non-cardiac gastric cancer: The first is related to the *Helicobacter pylori* (*H. pylori*) infection and atrophic gastritis. The second is related to gastroesophageal reflux- disease (GERD). The latter is also associated with esophageal adenocarcinoma (Compare et al., 2010).

2.2.1 *Helicobacter pylori* and its association with gastric cancer

Barry Marshall and Robin Warren were awarded the Nobel Prize in Medicine to identify *H. pylori*. Their findings have profoundly changed the diagnosis and treatment of the upper gastrointestinal disease associated with chronic gastritis. Earlier, lifestyle and anxiety were speculated to be the significant risk factors in peptic ulcer disease (Warren & Marshall, 1983).

H. pylori was classified as carcinogen group 1 by the International Agency for Research on Cancer of the World Health Organization (WHO) in 1994 (IRAC, 1994). Up to 80% of gastric ulcers are induced by *H. pylori* infection, and the inflammation caused by the bacterium predisposes to most gastric cancers (Karimi et al., 2014). However, in contrast, India, as one of the areas with high *H. pylori* infection rates, does not suffer from high gastric cancer incidence, which indicates that *H. pylori* infection is necessary but not sufficient for the development of *H. pylori*-associated GC. The interaction between *H. pylori* and other predisposing factors like genetics, the immune response of the host, and diet may explain these disparities (Graham, 2015).

H. pylori infection is strongly associated with intestinal-type GC development (Umeura et al., 2001). Gastric cancer risk increases with the more virulent infection and the more common strains of *H. pylori* worldwide; the cytotoxin-associated gene A (Cag A) *H. pylori* (Moss, 2016). *H. pylori* infection results in chronic inflammation,

intestinal metaplasia, and atrophic gastritis. Gastric atrophy and intestinal metaplasia of the gastric mucosal lining are collectively known as chronic atrophic gastritis (Raza & Bhatt, 2021). Patients suffering from severe chronic atrophic gastritis caused by *H.pylori* are at significant risk for intestinal-type GC (Park & Kim, 2015). The inflammation preceded by *H. pylori* infection leads to the high turnover of the gastro-epithelial cells, which leads to the accumulation of oxygen free radicals and nitrogen species increasing the risk of DNA damage and somatic mutations, thus promoting cancer development (Graham, 2015). These molecular changes are reversible after eradicating *H. pylori* (Graham, 2015; Muhammad & Eladl, 2019). The cascade of tumorigenesis in intestinal gastric cancer initiated by the *H. pylori* infection is illustrated in Figure 1.

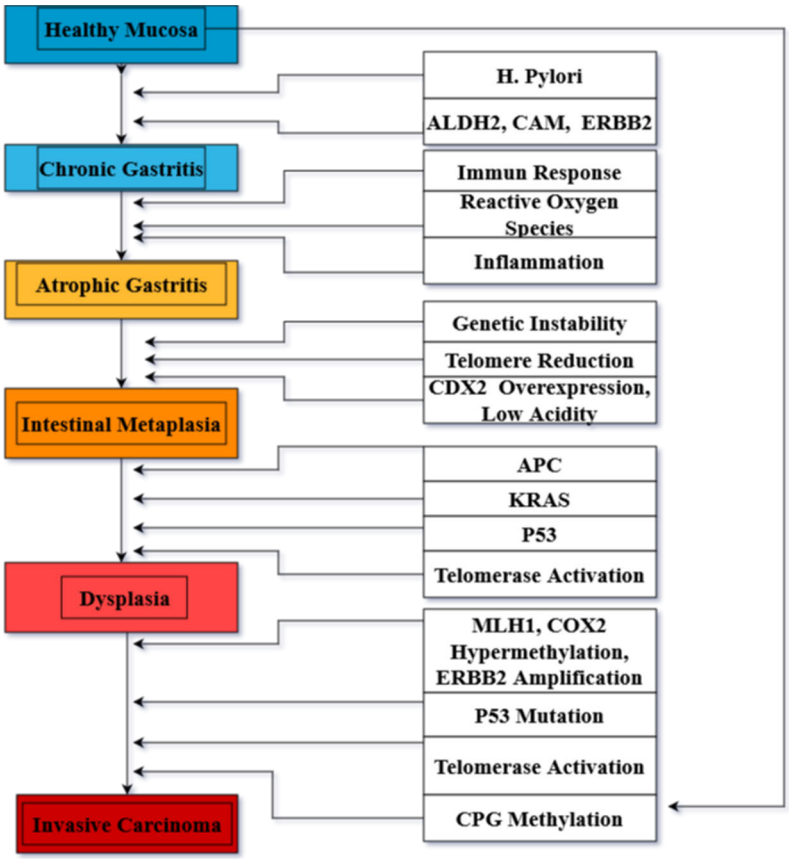

Figure 1. An intestinal-type gastric cancer carcinogenesis model includes the possible infectious and molecular etiological factors. In this model, the initial phase in the carcinogenic process is started by chronic inflammation. Chronic inflammation is associated with a classical histological cascade presented in colored boxes (left). The environmental factors, mutations, microsatellites and hypermethylation, and other signaling pathways implicated in the carcinogenic process are presented in uncolored boxes (right). Adapted from Poh et al. (2016)

2.2.2 Viruses and cancer

Cancer-causing viruses are commonly known as oncogenic viruses. It is estimated that approximately 12% of all human cancers may be connected to oncogenic viruses (Rositch, 2020). Oncogenic viruses have been classified as a group- 1 carcinogen by the International Agency for Research on Cancer (IARC) (IRAC, 2014).

2.2.2.1 Examples of oncogenic viruses and the general mechanism of their tumorigenesis

Oncogenic viruses include DNA and RNA viruses. Among the most common DNA viruses causing human cancers are *Epstein-Barr virus* (EBV), *human papillomavirus* (HPV), *hepatitis B virus* (HBV), *human herpes virus-8* (HHV-8), and *Merkel Cell Polyomavirus* (MCPV). In contrast, the most common RNA viruses attributed to human cancers are *hepatitis C viruses* (HBC) and *Human T lymphotropic virus type 1* (HTLV-1) (Moore & Chang, 2010; Chang et al., 2017). Table 1 shows oncogenic viruses and the associated tumors. Infection by oncogenic viruses is generally not sufficient for cancer development per se; additional factors, including host immunity, genetic predisposition, and somatic mutations, also play a role in neoplastic transformation (Mesri, 2014).

Table 1. Oncogenic viruses, associated cancers in human and genomic type

Oncogenic virus	Associated tumors	Genome
EBV	Burkitt's lymphoma, Hodgkin's lymphoma, immunosuppression-related lymphoma, T and NK cell lymphomas nasopharyngeal and stomach carcinomas	Double-stranded DNA Herpesvirus
KSHV	Primary effusion lymphoma and Kaposi sarcoma	Double-stranded DNA Herpesvirus
HPV HIGH-RISK (16,18)	Cervical, head and neck and anogenital tract carcinomas	Double-stranded DNA Papillomavirus
MCPV	Merkel cell carcinoma	Double-stranded DNA Polyomavirus
HBV	Hepatocellular carcinoma	Single-stranded and double-stranded DNA Hepadenovirus
HCV	Hepatocellular carcinoma	Positive-strand, single-stranded RNA Retrovirus

EBV = *Epstein Barr virus*, KHV = *Kaposi's sarcoma-associated herpesvirus*, HPV = *Human papillomavirus,* MCPV = *Merkel cell polyomavirus*, HBV = *Hepatitis B virus*, HCV = *Hepatitis C virus,* HTLV1 = *Human T-cell lymphotropic virus type 1.*

Many oncogenic viruses can induce persistent infections and chronic inflammatory responses in host cells. Viral persistence is not the only prerequisite for neoplastic transformation in virus-associated tumors; additional oncogenic hits are crucial for the complete transformation of the host cell (Krump & You, 2018). Oncogenic viruses rely on increasing the pool of infected cells by activating the cell proliferation mechanism. In contrast, acute infecting viruses increase the production of viral infectious particles (Morales-Pánchez & Fuentes-Pananá, 2014).

The neoplastic transformation by the oncogenic viruses can result from direct or indirect mechanisms. In the indirect mechanism, the oncogenic viruses can form an episome and be maintained as genetic elements within the host cell. The carcinogenic transformation triggered by immunosuppression or the oxidative stress resulting from chronic inflammation will continuously damage the local tissue; an example of an indirect oncogenic virus is EBV (Pierangeli et al., 2015). On the other hand, the oncogenic agents are detected in the direct mechanism as monoclonal forms and integrated within-host genom. Integrating the viral genome into the host genome triggers the host cells' transformation by downregulation of the tumor suppressor genes, like in *hepatitis B virus* (HBV) infection (Krump & You, 2018). However, some oncogenic viruses may require direct and indirect mechanisms to induce carcinogenesis (Morales-Sánchez & Fuentes-Pananá, 2014).

In addition, the oncogenic virus can provoke tumorigenesis by inducing genomic instability. Genomic instability could be in the form of gene amplification, gene deletion, aneuploidy, polyploidy, and chromosomal translocation (Korzeniewski et al., 2011). Furthermore, the oncogenic virus can induce loss of polarity and cell-cell contacts (James & Roberts, 2016). Essential signaling pathways manipulated by the oncogenic viral infection are the phosphatidylinositol 3-kinase–*AKT*–mechanistic target of rapamycin (*PI3K–AKT–mTOR*) and the mitogen-activated protein kinase (*MAPK*) pathways that mainly controls the transcription of genes that regulate cell proliferation and the immune response against viral (Niedźwiedzka-Rystwej et al., 2020; Morales-Sánchez & Fuentes-Pananá, 2014).

2.2.2.2 *Epstein-Barr virus* and cancer

Epstein–Barr virus (EBV), also known as HHV4 (*Human Herpesvirus Type 4*), is a linear double-stranded DNA virus detected in approximately 95% of adult populations worldwide (Stadtländer, 1999). However, a few proportions of EBV-infected individuals develop malignancy related to EBV (Tan et al., 2018). The life cycle of EBV is generally divided into two phases, lytic and latent phases. In the lytic phase (named "lytic" because the infected cells eventually lyse), after the viral RNA reverse transcription forms the virus DNA, the virus DNA integrates the host nucleus,

and new virus RNA is formed. During the lytic infection, all viral genes are expressed; some are supported the viral DNA replications and are known as early genes, for example, *BZLF1,* while the others are formed later and support viral particle formation and are known as late genes (Deng & Münz, 2021). When the surrounding environment is unfavorable, the virus enters the latency phases, and lytic replication is suspended until the virus senses the favorable environment (Song et al., 2019). During the latency phase, the host immune system cannot eliminate viral products (the Latency proteins) either because they are weakly immunogenic or corrupt the host immune responses; this results in persistent infection, which is, in turn, one of the critical features of the EBV infection in the cells (Murphy et al., 2009).

EBV's latent state is more often linked with EBV-associated malignancies; however, there is growing proof that EBV's lytic phase functions in EBV-associated tumorigenesis. The lytic phase in EBV infection acts in EBV-mediated oncogenesis by producing infectious viral particles and altering the host cell oncogenes. Furthermore, it is proved that an elevated viral infectious particle increases the risk of acquiring EBV-related cancer; because there are no EBV-positive tumor forms without EBV-infected precursor host cells. Thus, patients with high titer of EBV antibodies are at increased risk of developing EBV-associated cancer. In addition, the examination of EBV gene character in tumor samples of EBV-related tumors revealed some specific gene assays particularly elevated in these tumors. For example, the most common lytic gene measured in EBV-associated GC is the early gene *BZLF1* (Rosemarie et al., 2020).

The EBV-associated neoplasias are described by expressing various components of viral latencies (also known as transcripts). Most neoplastic cells in EBV-associated malignancies show a gene expression profile resembling that found in their non-neoplastic EBV infected cellular counterparts (Murphy et al., 2009). There are three latency types included in EBV infection; Latency type I, described in post-germinal center memory or proliferating B cells but also in B cell lymphomas. In Latency 1, the primary viral genes expressed are EBV nuclear antigen 1 (*EBNA1*), EBV-encoded small RNA (*EBERs*), BamHI-A rightward transcripts 0 (*BARF0*), and the latent membrane protein 2A (*LMP2A*). The Latency type II, described in the germinal center B cells, nasopharyngeal carcinoma (NPC), and classical Hodgkin lymphoma (HL), the primary viral gene expression includes *BARFs*, *EBERs*, *EBNA1*, and *LMPs 1*, *2A*, and *2B*. And lastly, the Latency type III, found in EBV-infected naïve B cells and activated B lymphoblasts, with the expression of all latent viral genes (including *EBERs*, *BARFs*, *EBNAs* (*3A, 3B, 3C*) and *LMPs* (*1, 2A, and 2B*). The most common protein expressed in all EBV–associated tumors is the *EBNA-1*. *EBNA-1* is expressed in Latency type III, II (Epithelial carcinoma and Hodgkin's lymphoma) and in Latency type I (Burkitt's lymphoma) (Crombie & Lacasce, 2019; Salamon et al., 2012*;* Kang & Kieff, 2015). The different Latency

types of EBV infection with the common Latency proteins in each Latency type, together with associated cancers, are shown in Figure 2.

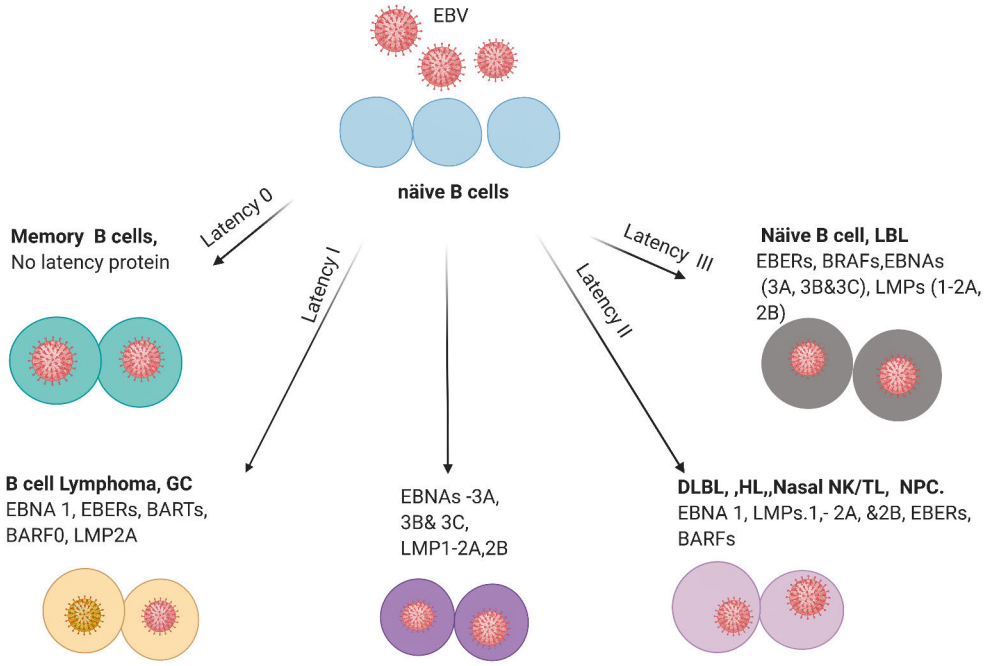

Figure 2. The different latency types of EBV virus with associated cancers include the possible latency proteins. DLBL = Diffuse large B cell lymphoma. HL = Hodgkin's lymphoma. NPC = Nasopharyngeal carcinoma. GC = Gastric cancer. LBL = Lymphoblastic lymphoma.

2.2.2.3 *Epstein-Barr virus* and gastric cancer

The *Epstein-Barr virus* is an important infectious agent implicated in gastric carcinogenesis. EBV infects 90%-95% of all adult population worldwide; however, EBV infection causes ~ 1% of all cancers. The risk of EBV as an etiological factor for cancer development likely results from a complex intersection of genetic, environmental, clinical, and dietary factors (Bakkalci et al., 2020; Takada, 2000).

In 1990, the association between EBV and gastric cancer with lymphoepithelial stroma was first detected using polymerase chain reaction (PCR) (Burke et al., 1990). However, in contrast to the universal association of EBV with nasopharyngeal cancer, EBV-related gastric cancer consists only of 9-10% of all gastric cancer subtypes (Murphy et al., 2009; Takada, 2000). The EBV+ gastric cancer subtype is characterized by molecular features, where the virus expresses restricted EBV latent genes belonging to Latency type 1;(*EBNA1, EBER, BARF0, LMP2A*). However, *EBER* is

the gold standard for detecting EBV in GC because it represents the *EBV DNA* genomes in the EBV+ GC samples (de Re et al., 2020). EBV-related latent genes are direct oncoproteins, which affect the host tumor suppressor genes and the cell cycle pathways (Yang et al., 2020; Strong et al., 2013).

2.2.3 Mismatch repair deficiency and microsatellite instability in gastric cancer

DNA damage can occur due to normal metabolic processes inside the cell or oncogenic viral infection plus an environmental factor. Different types of DNA damage can occur due to endogenous cellular processes like oxidation of bases, alkylation of bases (methylation), and mismatch of bases due to DNA replication errors. Typically, DNA damage occurs due to endogenous or exogenous (environmental) factors, which will trigger cell cycle checkpoint activation. The cell cycle checkpoint activation will lead to a pause in the cell cycle, mainly in G1 G2, and slow down the S-phase rate, providing the cell time to repair the damage before continuing division. If a cell cannot meet a particular requirement at a specific checkpoint, it moves to the G0 phase (Hustedt & Durocher, 2016). DNA mismatch repair (MMR) is a highly maintained repair pathway. MMR system consists of several proteins, including the products of *hMLH1*, *hMSH2*, *hMSH6*, and *hPMS2* genes. The MMR proteins function as dimers (*MSH2* and *MSH6*, *MLH1* and *PMS2*). The primary function of the MMR method is to identify the inconsistent nucleotides and stimulate other proteins to eliminate them and insert the correct nucleotide. Defects in the MMR system lead to the accumulation of small insertions/deletions in DNA microsatellite regions (Martín-López, & Fishel, 2013; Liu et al., 2008). Microsatellite instability (MSI) is one of the phenomena implicated in various cancers, including colorectal and gastric cancers; it was described initially in colorectal carcinoma (Leite et al., 2011; Ratti et al., 2018). MSI tumors have defective mismatch repair (MMR) and tumor suppressor genes, either due to germline mutation or epigenetic mechanism (Leite et al., 2011; Velho et al., 2014). Furthermore, MSI status is demonstrated by the early clinical stage and has a better prognosis than the non-MSI GC tumors (Zepeda-Najar et al., 2021).

Based upon the recommendations of a National Cancer Institute (NCI) workshop on MSI, MSI status is classified as high-level MSI (MSI-H), low-level MSI (MSI-L), or microsatellite stable (MSS) by using a panel of five microsatellites. The tumor is classified as MSI-H if two or more of the five markers show instability. In contrast, if one of the five markers shows instability, the tumor will be marked as MSI-L (Boland et al., 1998; Perucho et al., 1999; Luchini et al., 2019). The primary mechanism by which MMR system failure occurs among the MSI GC subtype is that

alterations occurring at the MMR system affect the *hMLH1* and *hMSH2* less frequently in *hMSH6* and *hPMS2* (Velho et al., 2014). However, the primary mechanism leading to MMR deficiency in sporadic and familial MSI GC cases is epigenetic silencing *hMLH1* by promoter hypermethylation (Leite et al., 2011).

Presently, several different methods are approved and currently in use to detect the MMR status in certain tumors, like colon, endometrium, and stomach cancers (Luchini et al., 2019); these methods include:

- Polymerase chain reaction (PCR) detects microsatellite sequences' amplification.
- Immunohistochemistry (IHC) staining to detect the expression of MMR proteins
- Next-generation sequencing (NGS) is used for the detection of MSI status.

The PCR method shows high sensitivity and specificity to identify the MSI status in a given tumor. By PCR, high-frequency MSI (MSI-H) status is provided if at least two of the five-microsatellite loci shift in size and show instability (i.e., have insertion/deletion mutations). In contrast, low-frequency MSI (MSI-L) status is given if the shift in size in one locus out of five, while the microsatellite stable (MSS) is given when cancer tissue is without any change compared to the normal one. More markers are needed to differentiate the MSS status and MSI-L in a given tumor tissue (Murphy et al., 2006).

On the other hand, the immunohistochemistry (IHC) method is indirect evidence of MSI and is used to score the IHC expression of MMR proteins. By the IHC method, the loss of expression of a single protein in the MMR proteins suggests that the tumor is MMR-D (Deficient). Thus, the tumor is MMR-P (Proficient) if the MSI status is examined and no defects in MMR protein expression can be detected. The IHC method showed concordant results in 95% of tumors, with a sensitivity of 82% and specificity of 98% compared to the PCR method (Shia, 2015). Moreover, the IHC method is affordable and available for routine work in pathology labs; it does not need paired tumors and normal tissue for the analysis.

Recently, MSI evaluation by next-generation sequencing (NGS) started to identify the MSI status in tumors. NGS-based methods computational algorithms allow MSI detection in thousands of microsatellite markers. In general, NGS-based techniques determine the correct sequence of nucleotides existing in a given DNA or RNA molecule. The discovery of NGS changes the standard of MSI detection in cancer. NGC covers a broader range of microsatellite loci; thus, it is not limited to the five microsatellite sites used in the PCR-based method. The PCR and the NGS methods are cost-effective and need paired tumor and normal tissue analysis. In addition, the NGS method is time-consuming and requires extensive validation with established methodologies (Ratti et al., 2018).

In this thesis, MSI status examined by the IHC methodology will be referred to as mismatch repair- deficient (MMR-D) and mismatch repair- proficient (MMR-P). On the other hand, the MSI status analyzed by different methodologies will refer to as microsatellite instability-high (MSI-H), microsatellite instability-low (MSI-L), or microsatellite stable (MSS).

2.3 Genetics of gastric cancer

2.3.1 Familial gastric cancer

One to three percent of gastric cancers are caused by hereditary defects. Familial gastric cancer (FGC) has been initially described in three Maori kindreds from New Zealand (Oliveira et al., 2004). The most common inherited mutation in FGC is in the E-cadherin (*CDH1*), and in Asian patients, *RHOA* gene mutations have been described. Another gene alteration that was also implicated in hereditary gastric cancer is *CTNNA;* however, the relevance is questionable (Petrovchich et al., 2016). Familial GC is characterized by diffuse histological type, epithelial-mesenchymal transition (EMT) phenotype, and poor prognostic clinical outcome. The only treatment option for patients with germline mutations *CDH1* is prophylactic gastrectomy for the high risk of diffuse-type GC (Guilford et al., 1998; Kakiuchi et al., 2014).

Lynch syndrome is an autosomal dominant syndrome with a mutation in DNA mismatch repair (MMR) genes. Lynch syndrome is characterized by a significantly high risk of colorectal, endometrial, and gastric cancer. Approximately 15% of GC seems to have microsatellite instability (MSI) associated with a mutation of the MMR genes (Latham et al., 2019).

A common form of familial adenomatous polyps –gastric cancer, similar to familial adenomatous polyposis coli (FAP), is an autosomal dominant disease caused by germline mutations in the adenomatous polyposis coli (*APC*) gene. Recently, published data showed GC had been cited as a cancer risk of FAP patients in the western population. However, less than 1% of FAP patients risk having cancer, indicating that it requires an environmental factor to transform the polyps into adenocarcinomas (Leone et al., 2019). Another rare syndrome is also related to *APC* mutation, referred to as Gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS). GAPPS is an autosomal dominant syndrome predisposing to GC (Rudloff, 2018). Moreover, a rare inherited gastric cancer can be seen associated with TP53 germline mutations in Li–Fraumeni syndrome (Masciari et al., 2011).

2.3.2 Chromosomal instability

Genetic instability is defined as a high frequency of mutations within the cellular genome. These mutations can include changes in nucleic acid sequences, rearrangements of the chromosomes, or aneuploidy. The most common genomic instability observed among human malignancies was chromosomal instability (CIN) (Giam & Rancati, 2015). The molecular mechanism in GC is still unclear; however, mitotic arrest and telomere attenuation, together with oncogene-induced replication stress and cell cycle checkpoint defects, could be possible scenarios (Maleki & Röcken, 2017). CIN can be analyzed at the molecular level by the arbitrarily primed polymerase chain reaction (AP-PCR). AP-PCR is a PCR-based DNA fingerprinting technique using primers whose nucleotide sequence is arbitrarily (randomly) chosen; this technique will examine the whole genome for possible alterations. In addition, other methods are used to analyze predefined genomic regions to detect the significant part in the chromosome damage, the loss of heterozygosity (LOH) analysis.

2.3.3 *CpG* island methylation in gastric cancer

One essential epigenetic alteration in cancer growth and development is the epigenetic silencing of tumor suppressor genes due to *CpG* islands' hypermethylation (Ottini et al., 2006). *CpG* islands are regions of the genome that contain a large number of *CpG* dinucleotide repeats. *CpG* island methylation phenotype positive (*CIMP*-positive) refers to concurrent hypermethylation in multiple loci in *CpG* island (Padmanabhan et al., 2017). The hypermethylation of gene promoters progresses with the histopathologic changes from chronic gastritis, intestinal metaplasia, adenoma, and carcinoma, suggesting a distinct pathway in gastric cancer development and progression (Figure 1).

2.4 Classification of gastric cancer

2.4.1 Anatomic classification of gastric cancer

Gastric cancer can be classified by anatomic location. In Siewert classification, GC tumors are classified concerning the gastroesophageal junction (GEJ) into distal esophageal carcinoma, gastric cardia, and sub-cardiac region (Siewert & Stein, 1998). Siewert classification is used in clinical practice for the surgical treatment decision in GEJ gastric cancers. Siewert type I covers tumors 1-5 cm above the EGJ. Siewert type II constitutes tumors 1 cm above and 2 cm below the EGJ. Siewert type III comprises tumors 2–5 cm below the EGJ. However, determining the exact site

of the squamocolumnar junction (Also known as Z-line) is sometimes problematic due to Barrett's esophagus (Kauppila & Lagergren, 2016).

Moreover, assigning the tumor's origin is frequently challenging, especially when the GEJ tumor has reached a considerable size. Therefore, the American Joint Commission on Cancer Classification (AJCC) proposed classifying tumors of the gastroesophageal junction and those involving the proximal 5 cm of the stomach as esophageal carcinomas. The tumor-node-metastasis (TNM) staging system has simplified the categorization of the tumors into either carcinoma of the esophagus and oesophagogastric junction or gastric carcinomas (Brierley & Gospodarowicz MK, 2017).

2.4.2 Histopathological classification of gastric cancer

Over a half-century ago, Pekka Laurén (Laurén, 1965) at the department of pathology, University of Turku, Finland, introduced the traditional histologic classification of GC, which still is broadly accepted and used by pathologists and physicians worldwide. The category includes two major histologic subtypes: intestinal-type and diffuse-type adenocarcinoma. The undetermined type formed a mixed histological type that can not be determined either intestinal or diffuse-type GC. The intestinal and diffuse types represent two different diagnostic entities; however, both types are associated with *H. pylori* infection. The intestinal type is more common and occurs in about 54% of the cases. It occurs twice as often in males as in females and is more common, especially in the antrum. Histologically, intestinal-type GC is characterized by malignant epithelial cells with cohesiveness and glandular differentiation infiltrating the surrounding stroma. Diffuse-type GC constitutes approximately 32% of the cases, is equally common in males and females, and is histologically formed of discohesive cells that infiltrate the surrounding tissue without glandular formation. Signet ring morphology is also a feature of diffuse-type GC (Laurén, 1965).

The World Health Organization (WHO) proposed the histopathological classification that categorized GC into four major histologic patterns: tubular, papillary, mucinous, and signet ring cell carcinoma, plus the uncommon histologic variants (Bosman, 2010). Less dominant histological elements often accompany the prominent pattern. The classification covers adenocarcinoma and all other gastric cancer types. Compared to Laurén's system, the tubular and papillary carcinoma in the WHO system roughly correspond to the intestinal types. In contrast, the discohesive with or without signet ring types is equal to the diffuse carcinoma in the Laurén system (Nagtegaal et al., 2020). Another histological classification, more complex and of limited use clinically, is the Goseki system. The Goseki divides GC based on the degree of tubular formation and intracellular mucin into four groups: Well-

formed tubules, poor intracellular mucin; well-formed tubules, rich intracellular mucin; poorly differentiated tubules, poor intracellular mucin; and poorly differentiated tubules, rich intracellular mucin (Goseki et al., 1992).

Although current histopathological classification systems influence surgical or endoscopic choices, they are still deficient in guiding precision personalized therapy for GC patients. New molecular classifications of GC have been introduced recently, and translational clinical studies are ongoing (Cisło et al., 2018).

2.4.3 Molecular classification of gastroesophageal adenocarcinoma

The Cancer Genome Association group (TCGA) and the Asian Cancer Research Group (ACRG) independently introduced a new molecular GC classification based on tumor molecular profiling. The TCGA consortium examined 295 gastric tumors and recognized four subtypes using detailed analyses of molecular data from six analysis platforms that included DNA sequencing, RNA sequencing, and protein arrays. The four molecular subtypes are EBV positivity, MSI status, CIN, or genomic stability (GS). On the other hand, the ACRG also classified gastric cancer into four subgroups: The MSI, MSS tumors showing EMT (MSS/EMT), MSS tumors with active TP53 (MSS/TP53+), and MSS with impaired function of TP3 (MSS/TP53-) (Cristescu et al., 2015). Both ACRG and the TCGA genomic subtypes were comparable and showed similarities: For instance, tumors with MSI were in both classification systems, and the TCGA GS, EBV+, and CIN subtypes were enriched in ACRG MSS/EMT and MSS/TP53− subtypes, respectively. The MSS/TP53+ is comparable with the CIN subtype. Compared to the ACRG classification, the TCGA classification was not correlated with survival. However, it provides essential evidence about the molecular profiling of GCs, including several genetic and epigenetic changes underlying gastric carcinogenesis. It can be helpful in the selection of the preferred therapy (Cristescu et al., 2015). However, and in contrast to breast cancer molecular classification, the new molecular classification of GC does not provide a clear path to novel treatment modalities. Not all new findings have been translated to clinical practice yet (Cisło et al., 2018). One of the remaining challenges is the lack of a robust genetic-clinical association. In the future, multidisciplinary committees are expected to include molecular classification in the personalized treatment decision of GC.

2.4.3.1 Characteristics of EBV, MSI, GS, and CIN subtypes of gastric cancer

Figure 3 shows the incidence of molecular subtypes with a summary of molecular profiles in the two critical molecular studies, the TCGA and ACRG. Compared with Lauren's classification, all MSI tumors in the TCGA study are intestinal-type adenocarcinomas, constituting 9% of gastric cancer cases. In contrast, the GS subgroup represents approximately 20% of the GC cases in the TCGA study and mainly represents diffuse-type adenocarcinomas (Bass et al., 2014).

Furthermore, a combining data from studies provided by the TCGA consortium and by Leung et al.(1999) on GC molecular subtypes showed that MSI GC subtype diagnosed at an older age (median age 72 years), located more frequently in the antrum (75%), and diagnosed at early stage with good prognosis. In addition, MSI GC is characterized by the lowest frequency of recurrence of the four GC subtypes; the same studies showed that MSI GC subtype showed a slightly higher prevalence in female patients (56%) and associated with *H. pylori* infection. (Leung et al., 1999; Bass et al., 2014). The MMR deficiency in the MSI GC subtype is mainly due to promoter methylation, which leads to transcriptional silencing of the DNA mismatch repair gene *MLHl* (Bass et al., 2014; Leung et al., 1999). Moreover, forty-two percent (42%) of tumors in the MSI GC subtype showed mutations in phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (*PIK3CA)* (Bass et al., 2014; Yamamoto & Imai, 2015).

The TCGA data showed that the absence of intestinal metaplasia characterizes the EBV+ GC subtype. EBV+ CG subtype is frequently located in the gastric fundus or body (62%) and is more frequent in male patients (81%) (Bass et al., 2014). Among all molecular subtypes, the EBV+ GC have the best outcome for both the recurrent-free survival (RFS) and the overall survival (OS) (Sohn et al., 2017); in addition, EBV+ GC had an elevated prevalence of DNA hypermethylation than any cancers reported by the TCGA study,(Bass et al., 2014). In addition, around 15% of the EBV+ tumors showed amplification of genes that encode *PD-L1* and *PD-L2* (Camargo et al., 2014; Bass et al., 2014)

Studies by the TCGA and others also yielded that the Genomic stable (GS) GC tumors are characterized by low mutation burden, frequent somatic *CDH1* mutations, and more frequent among the younger age group (median age 59 years) (Camargo et al., 2014; Bass et al., 2014).

The chromosomal instable (CIN) subtype constitutes 50 % of GC patients, about 73% of CIN tumors showed a high frequency of tumor suppressor TP53 mutations. TP53 subtype tumors are located more proximal and mainly in gastroesophageal junction/cardia (65%) (Bass et al., 2014). Esophageal carcinomas cannot be separated from the CIN GC subtype at the molecular level because both share the same molecular profile (Secrier et al., 2017).

Figure 3. Characteristics, common molecular alterations and frequency of molecular subtypes in GC. Results from TCGA, The Cancer Genome Atlas, and ACRG, Asian Cancer Research Group are presented. EBV = Epstein-Barr virus, MSI = microsatellite instability, GS = genomically stable, CIN = chromosomal instability. PIK3CA = Phosphatidylinositol-4, 5-Bisphosphate 3-Kinase Catalytic Subunit Alpha, KRAS = Kirsten rat sarcoma viral oncogene homolog, JAK2 = Janus-associated kinase 2, mTOR = Mechanistic target of rapamycin, PTEN = Phosphatase and tensin homolog, ARID1A = AT-rich interactive domain-containing protein 1A, PD-L1 = programmed death ligand-1, BCOR = B-cell lymphoma 6 corepressor, PI3K = phosphatidylinositol 3-kinase. RhoA = Ras homolog family member A, CDK = Cyclin-dependent kinase, CCNE1 = Cyclin E1, ROCK = Rho-associate protein kinase, ERBB2 = ErbB2 receptor tyrosine kinase 2, CCND1 = Cyclin D1, RTK = Receptor tyrosine kinase, CDH1 = E-cadherin, MSS/EMT = microsatellite stable/epithelial-mesenchymal transition, MSS/TP53+ = microsatellite stable/epithelial/TP53 intact. MSS/TP53- = microsatellite stable/epithelial/TP53 loss.

2.5 Treatment of gastric adenocarcinoma

To date, curative therapy for GC largely relies on the total resection of the tumor; thus, surgery offers the best chance of cure for GC patients (Johnston & Beckman, 2019). Total gastrectomy or subtotal gastrectomy combined with adequate lymphadenectomy remains the best chance for disease control (Giampieri et al., 2018). Endoscopic resection is an efficient approach modality for selected cases of early gastric cancer (Weledji, 2017).

Neoadjuvant chemotherapy, which refers to the preoperative chemotherapy used to treat a particular set of patients with locally advanced disease (tumors that reach/ or beyond the muscularis propria), was introduced after the publication of phase III randomized MAGIC clinical trial 2006 (Cunningham et al., 2006). Recently randomized, phase 2/3 trial was introduced by Al-Batarn et al. (2019); in this perioperative trial, the docetaxel-based triplet fluorouracil plus leucovorin, oxaliplatin, and docetaxel (AIO-FLOT4 trial) were used. This study emphasized the efficacy and safety of AIO-FLOT4 therapy for patients with advanced, resectable local gastric tumors. In general, the neoadjuvant treatment decreased the stage of the disease and improved progression-free and overall survival in patients with GC. However, the review by Ratti et al. revealed that patients with MSI-H tumors showed worse prognosis with the use of neoadjuvant chemotherapy; even though MSI status has a favorable prognosis, the poor prognosis reported after neoadjuvant chemotherapy of the MSI subtype GC suggests the harmful role of neoadjuvant drugs in this GC subgroup (Ratti et al., 2018). Thus, the analysis of the MSI status in GC has increasingly become essential.

In the Ascian population, it has been found that the optimal treatment strategy for GC patients with stage IIIA-IIIC disease is radical gastrectomy with D2 lymph node dissection (D2 includes all perigastric, coeliac, splenic artery, hepatoduodenal ligament nodes are systematically removed en-block with the stomach). Moreover, patients treated with radical gastrectomy and D2 lymph node dissection were found to have better OS and DFS after receiving adjuvant chemotherapy than those who did not (Chang et al., 2020). However, whether D2 lymphadenectomy is beneficial among the Western population or not is still under debate (Yarema et al., 2016; Kung et al., 2020).

Recurrence is not rare following operational resection for GC, even with negative margins in early-stage disease. Hence, the addition of chemotherapy and/occasionally radiotherapy is required for increased survival (Orditura et al., 2014).

There is increasing interest in the potential value of molecular subtypes for stratifying patients with gastric cancer. Studies reported that patients with the CIN molecular subtype had the most significant survival benefit with adjuvant chemotherapy compared to the GS subtype patients who had the least associated benefit with adjuvant chemotherapy (Smyth et al., 2016; Thiel & Ristimäki, 2015).

2.5.1 Targeted and immune therapies

Recently, immunotherapy for GC, including checkpoint inhibitors, has offered promising treatment options. Pembrolizumab is a checkpoint inhibitor that targets the PD-1/PD-L1 pathway approved for subsets of patients with advanced gastric or gastroesophageal junction cancer with PD-L1-positive tumors (Shitara et al., 2018; Seidel et al., 2018). Especially GCs of MSI-H and EBV+ molecular subtypes, with tumor-infiltrating CD8+ cytotoxic T lymphocytes and PD-1 expressing tumor cells, are good candidates for immune checkpoint inhibitors (Kim et al., 2019a). Other active immunotherapies include the chimeric antigen rector (CAR)-T cells approach and tumor vaccines. Both are being investigated in clinical trials to treat immunogenic GCs, the MSI-H, and EBV+ subtypes (Zhao et al., 2018; Panda et al., 2018).

A well-known targeted therapy is Trastuzumab. Trastuzumab was the first targeted treatment option. Trastuzumab combined with other chemotherapy to treat selected advanced gastric or gastroesophageal junction cancer patients with tumors showed elevated human epidermal growth factor receptor 2 (HER2, also known as ERBB2) expression (Apicella et al., 2017). Trastuzumab was FDA approved for GC treatment after the publication of Trastuzumab for Gastric Cancer (ToGA) phase III randomized controlled trial in 2010 (Bang et al., 2010). Another two-targeted therapy was recently introduced; Bevacizumab (vascular endothelial growth factor (VEGF) inhibitor) and Ramucirumab (vascular endothelial growth factor receptor (VEGFR2) inhibitor). These two drugs were a pioneering advance to targeting the tumor microenvironment and were reported to be a valuable approach for treating advanced GC with remote metastases. However, the development of chemoresistance towards Bevacizumab or Ramucirumab is common and may occur at an early phase of therapy (Itatani & Kawada, 2018).

2.6 Host immune response in cancer

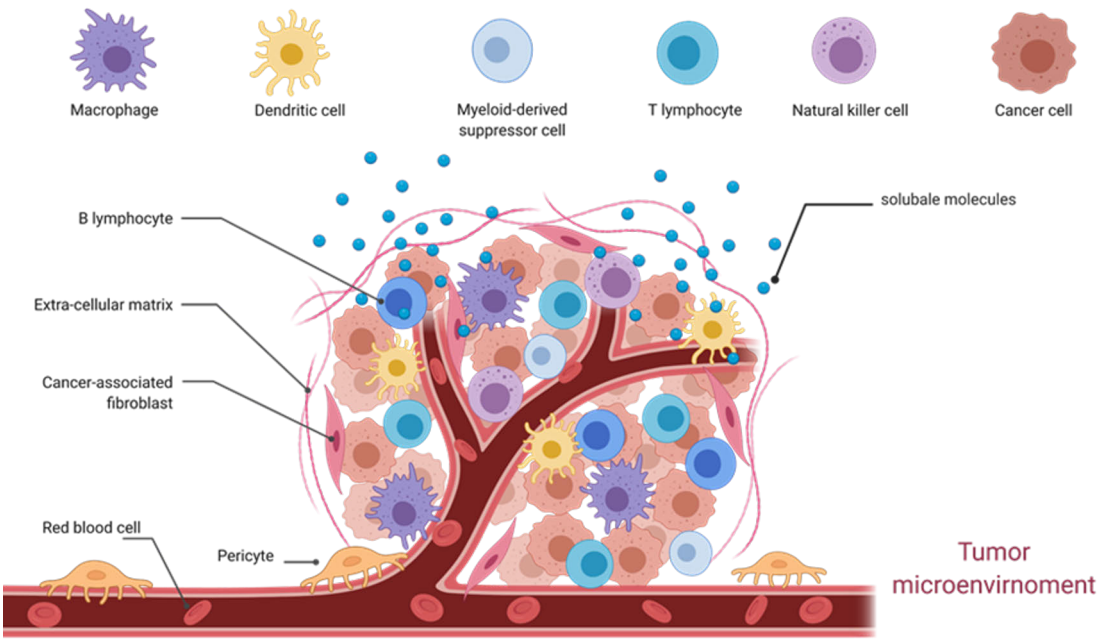

Figure 4. Components of the tumor microenvironment.

2.6.1 Cancer-associated lymphocytes and their role in cancer progression and prognosis

Cancer-associated lymphocytes (CALs) are an essential component of the Tumor microenvironment (TME), along with many other cell types (Figure 4). They participate in adaptive immunity and play a crucial role in cancer progression. However, cancer cells can escape the immune response and modify the microenvironment resulting in a situation in which immune cells can drive cancer progression and drug resistance (Galli et al., 2020). CALs (mainly T lymphocytes) have a unique function with inflammatory or anti-inflammatory consequences (Speiser et al., 2016). The T-cell receptor (TCR)-CD3 complex is expressed on all T lymphocytes. Cytotoxic T-cells express CD8, and helper T-cells express CD4 phenotype (Ostroumov et al., 2018)

Activated CD8+ T-cells can produce and secrete molecules that trigger target cells to undergo programmed cell death by apoptosis and kill infected cells after a recognition process involving the major histocompatibility complex class I molecule (MHC-Class I) (Zhu, 2018).

On the other hand, T regulatory cells (Tregs) are important with a phenotype; CD4+ T cell and CD25+. As defined by CD4 and CD25 expression, regulatory T lymphocytes comprise 5–10% of the mature $CD4^+$ T lymphocyte subpopulation. A subset of Tregs constitutes a specific population of Tregs, and they express the transcription factor forkhead box P3 (FOXP3). Tregs have a different function than T helper lymphocytes, and their role is to turn off the immune system response when it is no longer needed (Gol-Ara et al., 2012). FOXP3 can change naive T lymphocytes to regulatory cell phenotype, transcription factor forkhead box P3 (FOXP3) is considered the most specific Tregs marker so far (Hu et al., 2017; Hori et al., 2003).

Typically, the Tregs, including the FOXP3, protect hosts from the immune reactions mediated by CD8+ and CD4+ T lymphocytes, the immunosuppressive function of the Tregs prevents excessive damage to the normal tissue in the body, thus preventing allergic reactions, rejection of allograft, autoimmune disease, and graft versus host disease (Asano, 1996). Tregs suppress the immune response by several mechanisms, such as by producing anti-inflammatory cytokines or releasing molecules that kill activated immune cells or indirectly through inactivation of T lymphocytes via changing the function of dendritic cells (Schmidt et al., 2012). The immunosuppressive function of Tregs is well preserved in the TME and is effectively involved in tumor immune escape (Hu et al., 2017); however, the exact role of Tregs in tumor progression has yet not been fully discovered.

The presence of cancer-associated lymphocytes in the TME raises an important question: How do cancer cells avoid destruction by the immune system? Cancer cells escape the destruction by lymphocytes through two possible scenarios; either because of impaired function of the T lymphocytes due to the exhaustion of T lymphocytes or by getaway the recognition by the T lymphocytes due to the concept of immune surveillance. The following two chapters explain the two mechanisms in detail.

2.6.2 The concept of immune surveillance

Immune surveillance refers to a monitoring process of the immune system to detect and destroy the infected cells and the neoplastic cells in the body. Lewis Thomas and Frank Macfarlane Burnet formulated the immune surveillance hypothesis in the 1950s (Ostroumov et al., 2018; Thomas, 1982). According to this hypothesis, the immune system can eradicate tumor cells; however, tumor cells can edit the role of T lymphocytes and escape immune destruction.

A study by Robert Schreiber entitled "Three Es of cancer immune-editing" suggested. In this theory, tumor cells avoid immune elimination via a complicated process in which the interaction between tumor cells and the CALs passes through three phases. The first phase is the immune eliminating phase, where the tumor cells can

be destructed and eliminated. The second phase is the immune equilibrium phase, which leads to an equilibrium state between tumor cells and CALs. Finally, the immune escape phase is where tumor cells evade and escape immune destruction (Dunn et al., 2004b). According to the cancer immune-editing theory, some tumors grow faster, reach the" escape phase," and are manifested clinically as primary or early metastatic tumors.

Typically, the host protective role is initiated when the host's immune system recognizes foreign antigens expressed by the tumor cells; the host's immune cells eliminate the tumors before they become clinically manifest. Some subsets of solid cancers showed prominent immunogenic tumor antigens referred to as neoantigens. Neoantigens are tumor-specific antigenic peptides that may be produced following germline mutations like in melanomas (Van Allen et al., 2015) or frameshift mutations in MSI-H tumors (Ratti et al., 2018). In addition, oncogenic viruses, including Epstein–Barr virus (EBV), can encode neoantigens (Qin et al., 2019; Yang et al., 2020). Neoantigens are ideal immunotherapy targets because the host immune system recognizes them as non-self-antigens. In theory, CD8+ T lymphocytes recognize tumor neoantigens, trigger the cytolytic activity, and kill cancer cells. However, tumor cells have derived specific mechanisms that turn off neoantigens' immunogenicity, thereby escaping immune surveillance. These mechanisms include loss of neoantigens by chromosomal instability–induced copy number alterations so that tumor cells become invisible to the host immune system, or at the RNA level, neoantigens expression can be decreased by promoter hypermethylation (Jiang et al., 2019). In addition, in many cancer types, including GC, tumor cells can escape the immune surveillance by downregulation of tumor-neoantigen-specific-T-lymphocyte receptors (TCRs), impairing the antitumoral response of reactive T lymphocytes, and inducing apoptosis of lymphocytes (Dunn et al., 2004a).

2.6.3 The development of cancer-associated T lymphocytes exhaustion

Studies showed that T lymphocytes were not as effective against cancer as expected, and the possible reason could be partially due to T lymphocyte exhaustion or dysfunction (Zhang et al., 2020). The mechanisms of T lymphocyte exhaustion are complicated and yet not fully discovered. However, the mechanism of lymphocyte exhaustion is mainly characterized by the absence of inflammatory mediators and inhibitory mechanisms; thus, T lymphocyte exhaustion in cancer differs from chronic infections. Furthermore, one of the different inhibitory mechanisms linked to T lymphocyte exhaustion is the presence of T lymphocytes with specific self-regulatory antigens (Davoodzadeh et al., 2017).

Researchers found that the molecular suppressive mechanisms of the T lymphocytes started by up-regulated expression of inhibitory receptors leads to reduced cytotoxic activity and decreased T lymphocytes' effective cytokine production (Davoodzadeh et al., 2017). Pandya et al. proved that altered signaling pathways on CALs in the TME help produce a suppressive inhibitory type-TME, resulting in exhausted T lymphocytes (Pandya et al., 2016). At the same time, other researchers suggest that exhaustion of the CALs could be due to impaired memory T helper lymphocytes which is a subset of CD8+ cells (Jiang et al., 2015).

The studies of the concept of T lymphocyte exhaustion lead to the development of a technique to retain the function of T lymphocytes. In this approach, a patient's T lymphocytes are altered in the laboratory by adding a particular receptor is called a chimeric antigen receptor (CAR). This receptor can track cancer cell antigens, then the modified T cells or the CAR-T lymphocytes, regiven to the same patient by infusion. This type of treatment makes the activated T lymphocytes engaged in the combat against cancer (Zhang et al., 2017).

2.6.4 The prognostic values of intratumoral T lymphocytes

The immune cells have been implicated in cancer behavior, affecting all cancer growth and development stages. Recently, studies utilizing the histopathological examination of TME in various solid tumors, including GC, revealed that high numbers of T lymphocytes infiltration had been associated with better survival regardless of the clinical stage (Galon et al., 2006; Kemi et al., 2020). High CALs are robust prognostic predictors over the MSI status in colon cancer (Mlecnik et al., 2016). In addition, the amount of intratumoral T lymphocytes is associated with a better disease outcome in various human cancers; like ovarian cancer (Li et al., 2017), melanoma (Fu et al., 2019), breast cancer (Gao et al., 2020; Meng et al., 2018), lung cancer (Kinoshita et al., 2016), pancreatic cancer (Tahkola et al., 2019), and colorectal cancer (Zhao et al., 2019).

2.6.5 Host Immune response in EBV+ gastric cancer

The study of the TME in the samples of EBV+ GC tumors revealed that it is the most extensive immune infiltrated tumor (Gong et al., 2019; Wee et al., 2018). The standard CALs in EBV+GC are the CD8+ cytotoxic T lymphocytes (Ratti et al., 2018). TME in EBV+ GC also showed marked differences compared to the TME of EBV-GC; however, both the immune activation and immunosuppression mechanisms coexist in the EBV+GC. Researchers found that the proportion of immune activation molecules (like granzyme B, FasL, TNF-α) is higher in the TME of EBV+GC than

EBV- GC. (Gong et al., 2019). On the other hand, the immunosuppression molecules as the *IL-1β* gene encodes *interleukin-1β (IL-1β*) are also highly expressed in the EBV+GC TME. The *IL-1β* recruits large numbers of nonspecific lymphocytes, which inhibit direct contact between the tumor cells and EBV-specific cytotoxic T lymphocytes, thus, preventing the cytotoxic effect of CD8+ T lymphocytes (Mbongue et al., 2015; Nishikawa et al., 2018).

In addition, the tumor cells escape the eradication by the high numbers of CD8+ T lymphocytes because of a high level of IFN-gamma expression, which leads to indoleamine expression 2, 3-dioxygenase (*IDO1*), a potent immune cell inhibitor. The IDO1 expression can explain the relative resistance of tumor cells in EBV+ GC to the high numbers of cytotoxic immune cells (Mbongue et al., 2015; Nishikawa et al., 2018).

In addition, the PD-1/PDL1 interaction plays a crucial role in immune escape and has key data for target immunotherapy. Among the EBV+ GC, tumor cells are associated with frequent recurrent amplification of the 9p24.1 locus containing the CD274 gene encoding PD-L1. PD-L1 interacts with the co-inhibitory molecule programmed death receptor-1 (PD-1) expressed by T lymphocytes. The increased expression of PD-L1 is associated with the EBV+ status, and studies revealed that patients with increased PDL1 have a favorable prognosis (Cho et al., 2016; Nakano et al., 2021).

2.6.6 Host immune response about microsatellite instability status in gastric cancer

The microsatellite instability (MSI) phenotype is strongly linked with immune cell signaling. The association of the MSI-H phenotype with high numbers of infiltrating immune cells is explained by the accumulation of frameshift mutations and the synthesis of neoantigens that trigger the host immune system (Ratti et al., 2018). The study of TME of the MSI GC subtype revealed high numbers of CD8+ T lymphocytes and expressed elevated values of PDL1 (Cho et al., 2018). However, different intratumoral T lymphocyte infiltration levels were detected among the MSI subtype GC. According to the level of tumor-infiltrated lymphocytes and the expression of PD-L1, researchers classify TME in the MSI GC into three types: 1). Type I, (PDL1+TIL+), 2). Type II (PDL1-TIL-), 3). Type III (PDLI+TIL-) and type IV (PDL1-TIL+), in general, type-I GC (PD-L1+ TIL+) are the most common type, and 70% belong to the MSI-H subtype. According to this classification, patients with type I TME would be a good candidate for checkpoint blockade therapy (Puliga et al., 2021; Cho et al., 2018).

Despite the large amounts of cytotoxic T lymphocytes in the MSI tumors, tumor cells are not naturally eliminated. The possible reason could be that high numbers

of the favorable T lymphocytes (CD8+ T lymphocytes) are paralleled by the unfavorable infiltration of Tregs (FOXP3+ T lymphocytes); or due to T lymphocyte's function exhaustion. T lymphocytes' unresponsiveness and high expression of immune checkpoint markers, the PD-1, and PD-L1 in MSI GC subtype explain why patients with MSI-H GC showed only a marginal survival benefit despite a rich infiltration of tumor-infiltrating cytotoxic T lymphocytes (Lazăr et al., 2018). Nevertheless, patients with MSI-H phenotype could be good candidates for immune checkpoint blockade therapy.

2.7 Actin cytoskeleton and its role in cancer

The actin microfilament system is the powerhouse of cellular motility and migration, and actin is the most abundant intracellular protein in eukaryotic cells; it provides a cytoskeleton, gives architectural support, and interferes with adhesions; it also forms the skeleton of cellular protrusions used by the immune cells while migrating towards the infected tissue (Paul & Pollard, 2009; Svitkina, 2018). The actin cytoskeleton comprises actin polymers formed from globular actin (G-actin) subunits (Ridley & Hall, 1992).

The formation of a new actin filament starts with two or three actin monomers forming an actin core or nucleus with the help of actin nucleation factors. Filament elongation can occur spontaneously once the nucleation has occurred and stabilized (Pollard & Borisy,2003; Bartolini et al., 2010). Actin filaments are polarized structures with pointed and barbed ends, and polymerization is inducted by uncapping the positive ends (the barbed ends). Actin monomers are attached instantly at the barbed end and gradually at the pointed end. The availability of free actin monomers defines the actin's spontaneous elongation rate (Ridley& Hall, 1992). Barbed end elongation can be promoted by the formin proteins (Gomez et al., 2007) or stopped by capping proteins. The process of polymerization of unbranched actin filaments is illustrated in Figure 5.

An essential feature of cancer cells is their ability to migrate and develop distant metastasis, which may be facilitated by abnormal expression or regulation of actin cytoskeleton components. For instance, actin-associated proteins, including WASP (Wiskott-Aldrich syndrome protein), SATB1 (Special AT-rich Binding protein 1), villin, and nesprin proteins, are involved in all steps of carcinogenesis (Izdebska et al., 2020). However, information on the specific cytoskeletal alteration in GC is sparse.

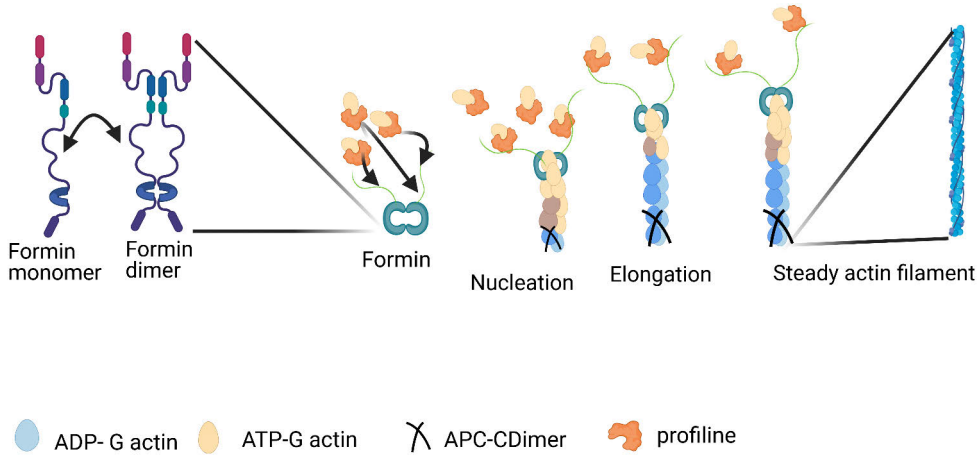

Figure 5. Formin is the leading molecule in actin polymerization. The formation of new actin started by nucleation by actin monomers. Nucleation and elongation factors utilize Profilin-bound actin monomers, including the formin dimer. The actin monomers are added quickly at the barbed end (the positive end) and slowly at the pointed end (the opposing end). The tumor suppressor protein Drosophila homologs of adenomatous polyposis coli -C (APC-C) dimer act as an actin assembly-promoting factor and are attached directly to filamentous actin.

2.7.1 The Formin proteins

Formins are a group of large multidomain proteins preserved in all eukaryotic organisms. Formins are characterized by a highly conserved formin homology 1 (FH1) domain and formin homology 2 (FH2) domain. Both domains are involved in reconstructing the actin cytoskeleton (Castrillon & Wasserman, 1994). The FH2 domains accelerate linear elongation of actin filaments by associating with their growing barbed positive ends via protection against capping proteins (Pollard & Borisy, 2003). While the FH1 domain mediates the recruitment of profilin-bound actin monomers for actin nucleation, it is also involved in the linear growth of the actin filaments. The formation and elongation of actin filaments depend on the availability of free actin monomers and the ability of formins to constitute the linear growth actin filaments. Besides the FH1 and FH2 domains, formin contains other regulatory domains responsible for activation and autoinhibition of the formin molecule. According to the presence or absence of the regulatory domains, the formins family are divided into diaphanous related formins (DRFs) and non-DRFs (Bogdan et al., 2013; Randall & Ehler, 2014). The commonly investigated formins are the DRFs subtype, while the non-DRFs subtype is still largely uncovered. Most of the DRFs activated

by small GTPases of the Rho superfamily and commonly formed of the following regulatory domains:

- A GTPase binding domain (GBD)
- A diaphanous inhibitory domain (DID) near the N-terminus
- A small diaphanous autoregulatory domain (DAD) close the C-terminal

The interaction of DAD and DID results in the stop of the nucleation and acceleration of actin by masking the FH2 domain, leading to an inactive state of the DRFs (Bartolini & Gundersen, 2010). The non-DRFs group is less characterized and lacks the N terminal GBD and DID (Goode & Eck, 2007). Figure 6 shows the structure of DRFs formin monomer and the mechanism of the Actin nucleation process.

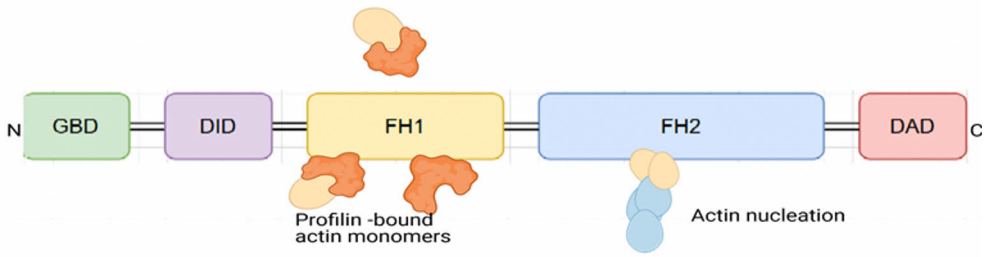

Figure 6. Schematic illustration shows formin protein monomer and actin filament assembly**.** The arrangements of domains that characterize the DRF family are GBD = GTPase binding domain, DID = Diaphanous inhibitory domain, FH1 = Formin homology domain 1, FH2 = Formin homology domain 2, DAD = Diaphanous autoregulatory domain. FH1 domains recruit profilin–actin complexes and accelerate the addition of actin monomers to the FH2-barbed end. FH2 domain is responsible for the actin nucleation.

There are fifteen formin genes in mammals (Higgs & Peterson, 2005). Formins have different functions, variable subcellular locations, and expressions in the different cell types (Bogdan et al., 2013). Several formins regulate the formation of cellular projections and stress fibers, migration, and adhesion (Homem & Peifer, 2008). Other functions of formins include regulating microtubules in both interphase and mitotic spindles (Bartolini & Gundersen, 2010). The DRFs and non-DRFs formins with their subcellular localization and function within the cell are illustrated in

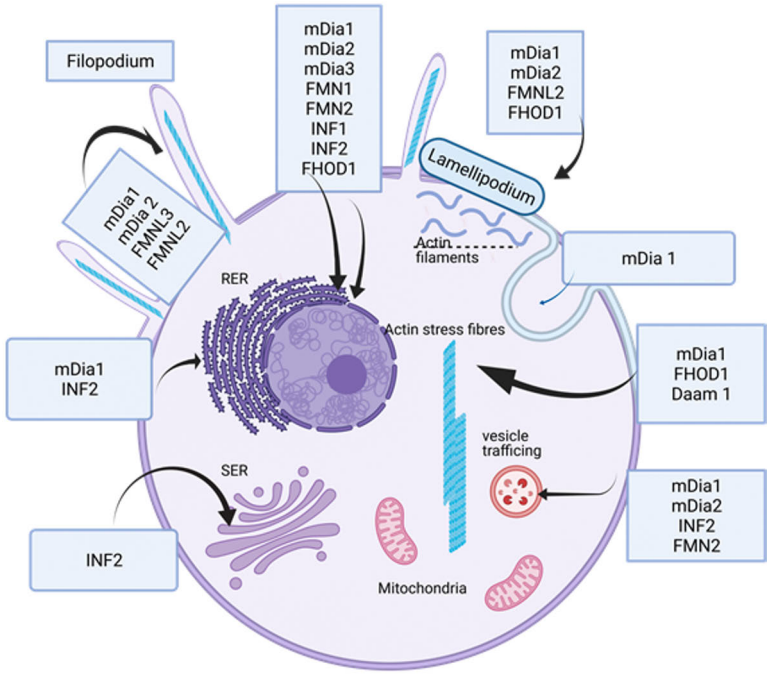

Figure 7. Formin types. Their different subcellular locations and localized actin assembly**.** Each formin type has a specific place and function in the cells. The DRFs group includes Diaphanous-related formin 1, 2, 3. (mDia1, 2 and 3 also known as DRF1,2, and 3), Disheveled association activator of morphogenesis 1, 2 (DAAM1 and DAAM2), formin-like protein 2 (FMNL2, also known as FRL3), formin-like protein 3 (FMNL3 also known as FRL2), FH1/FH2 domain-containing protein 1,3 (FHOD1 and FHOD3). The non-DRFs group includes Fomrin-1, 2 (FMN1, 2) inverted formin1, 2 (INF1, INF2). RER, rough endoplasmic reticulum. SER, smooth endoplasmic reticulum.

2.7.2 Formins in human disease, including cancer

Experiments have revealed that mutations in formin genes cause severe cell and tissue morphogenesis, cytokinesis, and cell polarity (Jackson-Grusby et al., 1992). Despite the significant discoveries on formins' role in cell biology and embryogenesis, few studies have directly implicated a causative role in disease pathogenesis. An autosomal dominant non-syndromic deafness (DFNA1) was the first hereditary disease linked with a mutation in a formin gene (Lynch et al., 1997). The condition is marked by severe hearing loss and complete deafness by 30 and is caused by frameshift mutations of the *DIAPH1* gene (DRF1 formin), leading to disturbed cytoskeletal function in the inner ear hair cells. In addition, *DAIPH2* gene (DRF2 formin) translocation is associated with familial premature ovarian failure disease (Bione et al. 1998), while the Charcot-Marie-Tooth neuropathy is characterized by chronic neuropathy and focal segmental glomerulosclerosis (FSGS) was linked to INF2 mutation (Jin et al., 2015). Microdeletion of one or more genes in the 2q23.1 harboring FMNL2 might be responsible

for the standard phenotypic features such as short stature, microcephaly, and mental retardation in humans (Milani et al., 2015).

In cancer, experimentally, knockdown of mDia markedly reduces the invasive activity of cultured breast cancer cells by altering the extracellular matrix's degradation mechanisms (Kim et al., 2016a). In addition, high FMNL2 and FMNL1 expression correlate with metastasis and the existence of epithelial-to-mesenchymal transformation (Zhu & Ding, 2008; Gardberg et al., 2014). The high FMNL1 expression was reported in the basal breast cancer cell line; basal breast lines are a standard EMT model associated with poor prognosis (Gardberg et al., 2014). In contrast, the worse outcome in hepatocellular carcinoma was associated with low expression of FMNL1 (Liang et al., 2011).

Information regarding the effect of formins in GC development and progression is rather limited. Wang et al. found a significant association between the elevated formin proteins and invasion, distal metastases, and gastric cancer progression. According to their study, FMNL1 is one of five core genes correlating with pathological stage and lymph node metastasis in gastric cancer (Wang et al., 2020a). On the other hand, the silencing of FMNL2 in experimental models inhibits tumor progression and metastasis in gastric cancer cells (Zhong et al., 2018). Recently Liang et al. (2017) investigated the role of FHOD1 in cancer progression in GC cell lines; this study revealed that FHOD1 is one of the critical genes linked with advanced disease and metastasis in GC patients (Liang et al., 2017). More recently, FHOD1 was also studied in GC clinical samples and was found to be elevated upon tumor progression and experimentally act as an important promoter of the proliferation and invasion of gastric cancer cell lines in the same study (Jiang et al., 2021). In summary, while there is experimental evidence linking formins to GC biology, their expressions in clinical GC specimens and their association with the outcome are still primarily uncovered.

In this thesis book, we have focused on two DRFs formin proteins: FMNL1 and FHOD1. These two formins were chosen because previous literature has indicated their role in the progression and metastasis of cancer cells *in vitro*. IHC analysis found an interesting IHC profile of FMNL1 and FHOD1 in normal and cancer tissue clinical samples (Gardberg et al., 2014).

2.7.2.1 FMNL1

Human leukocyte formin, or FMNL1, is mainly expressed in lymphoid tissues and hematopoietic tissues (Krainer et al., 2013).FMNL1 is connected to many immunological functions. For instance, FMNL1 has a migratory role in macrophages, and it is a crucial controller of podosomes (an adhesive structure in macrophages) (Miller & Blystone, 2015). Knockdown of FMNL1 resulted in the compromised phagocytotic activity of the phagocytic cells (Seth et al., 2006; Naj et al., 2013). FMNL1 is

overexpressed in non-Hodgkin lymphoma and leukemic cell lines (Favaro et al., 2006; Schuster et al., 2007; Favaro et al., 2013). Interestingly, FMNL1 is expressed in many epithelial cancer cell lines and clinical samples; it is linked to cancer outcomes in cancer. FMNL1 was upregulated in basal breast cancer (Gardberg et al., 2014). In non-small cell lung cancer (NSCLC), FMNL1 expression was associated with decreased bone metastasis (Yang et al., 2019), while in glioblastoma multiforme, upregulation of FMNL1 was detected as an unfavorable prognostic factor (Higa et al., 2019); in leukemia, FMNL1 enhances the generation and migration of leukemia cells (Favaro et al., 2013). A recent study showed that FMNL1 expression was reported in advanced high-grade GC tumors, and upregulation of FMNL1 transcription levels was associated with unfavorable OS in GC patients (Nie et al., 2020). These observations suggest that upregulated FMNL1 may have a role in malignant transformation and prediction of cancer outcomes. However, comprehensive investigations of FMNL1 in GC clinical specimens are still lacking.

Structurally, FMNL1 belongs to the DRFs group of formins. Three known FMNL1 isoforms differ in their C-terminus, referred to as the FMNL1, 2, and 3 (Young & Copeland, 2010). FMNL1 has a weak actin nucleation activity, and its primary function is to elongate and bundle the actin filaments (Harris et al. 2006). FMNL1 acts with the other formins in controlling various cellular processes of T lymphocytes, some cellular processes that affect the cellular cytotoxicity activity of the T lymphocytes (Gomez et al., 2007; Andrés-Delgado et al., 2012). Upregulation of FMNL1 was associated significantly with tumor immune infiltrations, mainly CD8+ cytotoxic T lymphocytes; this indicates that the expression of FMNL1 might modulate tumor immunity by regulating the infiltration of cancer-associated lymphocytes (Colón-Franco et al., 2011).

2.7.2.2 FHOD1

Formin homology 2 domain-containing protein 1 or FHOD1 belongs to the DRFs formin family. The exact role of FHOD1 in tumorigenesis and cancer progressions is not known. However, some research has revealed an essential correlation between upregulation of FHOD1 and epithelial-to-mesenchymal transition (EMT). EMT is a necessary mechanism for cancer cells to facilitate metastasis and invasion by enhancing cellular motility, triggering stem-cell characteristics, and arresting senescence and apoptosis (Nantajit et al., 2015). FHOD1 is primarily expressed in mesenchymal cells and is specifically upregulated upon EMT. Thus, FHOD1 may be required to maintain mesenchymal phenotype, migration, and invasion of tumor cells (Gardberg et al., 2013; Jiang et al., 2021).

Some reports have examined FHOD1 concerning cancer. In cultured breast cancer, melanoma, and squamous cell carcinoma cells, knockdown of FHOD1 reduced

cancer cell movement and invasion (Peippo et al., 2017; Gardberg et al., 2013; Heuser et al., 2018). The knowledge about the association of FHOD1 and GC is scarce. Recently Jiang et al. (2021) published a study on gastric cancer tissues. FHOD1 expression in GC clinical samples was significantly increased compared to non-neoplastic adjacent tissues. In addition, the higher expression of FHOD1 was associated with reduced overall survival in the same cohort, indicating that FHOD1 could be a prognostic indicator in GC. Nevertheless, the exact predictive association of FHOD1 in GC still needs further analysis.

Structurally, FHOD1 contains similar domains as other DRFs family members, the FH1, FH2, DAD domains, and DAD-mediated autoregulation. However, compared to the other DRFs family, the N terminal region of FHOD1 is different. The activation of the GBD region occurs due to interactions with Rac-Ras-GTPase instead of Rho-GTPases. The release of the inactive state (autoinhibition) FHOD1 is unique. It differs from the rest of DRFs members, and it starts by phosphorylation of serine and threonine residues in the C-terminal DAD by ROCK (Schulze et al., 2014). Figure 8 illustrates the structure of the FHOD1 formin protein and its activation process. Functionally, FHOD1 does not involve actin nucleation and elongation, a common feature of the DRFs family members. Instead, FHOD1 serves as an actin-bundling protein, assisting the formation of thick F-actin bundles. In addition, FHOD1 is involved in budding, actin arcs, and more mature stress fibers; it was also found in adhesions in cultured cells (Schulze et al., 2014).

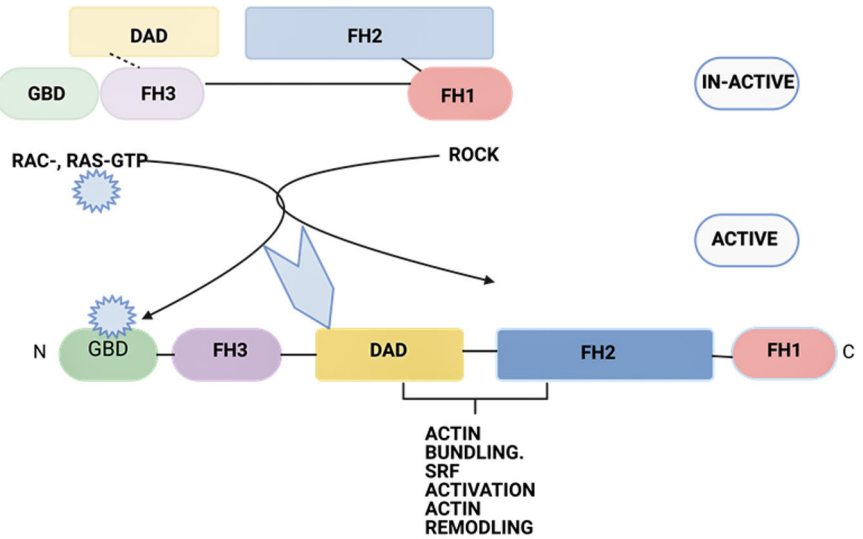

Figure 8. The structure of the FHOD1 formin proteins. The autoinhibition starts with the activation of the ROCK molecule toward the C terminal. The release of Rac-, Ras-GTP activates the GBD domain. The activated FHOD1 protein enhances actin bundling and remodeling with activation of SRF, which activates the cytoskeleton-associated genes.

3 Aims

This study concentrates on the expression of specific biological markers and their correlation with the prognosis and progression of gastric cancer.

The specific aims of this study are:

1. To explore whether IHC/ISH-based analysis of EBV expression, MSI -status, and E-cadherin and TP53 expression pattern recapitulates the gastric cancer molecular subtypes and demonstrates prognostic correlations in intestinal gastric cancer.
2. To study the presence of T-lymphocyte subtypes, mainly CD3, CD8, and FOXP3 T-lymphocytes in distinct molecular subtypes of intestinal-type gastric cancer, and evaluate their predictive correlations.
3. To study whether FHOD1 and FMNL1 formin proteins are expressed in gastric cancer molecular subtypes and whether their expression is associated with clinical features and outcomes.

4 Materials and Methods

4.1 Patient and tissue material (I–III)

The studies included in this thesis (I–III) were retrospective and had histopathological tissue material from a total number of 244 patients treated at Turku University Hospital in the years 1993-2012. All patients were diagnosed with adenocarcinoma of the stomach, gastroesophageal junction, or distal esophagus. Tumor tissues were re-assessed histologically by an expert pathologist (NM) to confirm the presence of adequate representative tumor tissue of both intestinal (N=190) and diffuse GC types (N=152). All intestinal tumor samples (N=190) were primarily included in the next generation (ng)TMAs, and tissue material from consecutive diffuse-type gastric adenocarcinoma of 54 patients was used as a control in the same ngTMAs. All studies (I–III) were focused on intestinal-type gastric carcinoma. The detailed selective inclusion and exclusion criteria for our tissue samples are described in detail in the original article I.

Among all patients, 11.9% (29/244) received preoperative chemotherapy (13 patients with intestinal-type and 16 with diffuse-type tumors). Patients who had received perioperative chemo-radiotherapy were excluded from the studies because preoperative therapy resulted in inadequate surgical material for immunohistochemical analysis. Information regarding the *Helicobacter pylori* status was available for 78/190 patients of intestinal-type gastric cancer, of which 20/78 were positive for H. pylori. The tumor stage was assessed according to the WHO Classification manual 2010 (Bosman et al., 2010). The re-evaluation of the staging was carried on according to the manual TNM staging system, eighth edition for study III (Brierley, Gospodarowicz, 2017). The manual TNM staging system, 8th edition, is summarized in Table 2. According to the current guidelines, study I–III reporting has been performed (Sauerbrei et al., 2018). The clinical characteristic of patients and tumors included in the original publications I–III are presented in Table 3.

Table 2. The AJCC TNM Classification system and corresponding staging group, 8th Edition.

Stage	T	N	M
IA	T1	N0	M0
IB	T2	N0	M0
	T1	N1	
IIA	T3	N0	M0
	T2	N1	
	T1	N2	
IIB	T4a	N0	M0
	T3	N1	
	T2	N2	
	T1	N3a	
	T1a	N3b	
IIIA	T4b	N0	M0
	T4a	N1	
	T3	N2	
	T2	N3a	
IIIB	T4b	N1	M0
	T4b	N2	
	T4a	N3a	
	T3	N3a	
	T2	N3b	
	T1	N3a	
IIIC	T4b	N3a	
	T4b	N3b	
	T4a	N3b	
	T3	N3b	
IV	Any T	Any N	M1

AJCC. American Joint Committee on Cancer, T1. Tumor invades lamina propria, muscularis mucosa, or submucosa, T2. Tumor invades muscularis propria, T3. Tumor penetrates subserosal connective tissue without invading visceral peritoneum or adjacent structures, T4a. Tumor invades serosa (visceral peritoneum), T4b. Tumor invades adjacent structures, N0. No regional lymph node metastasis, N1. Metastasis in 1–2 regional lymph nodes, N2. Metastasis in 3–6 regional lymph nodes, N3. Metastasis in 7 or more regional lymph nodes, N3a. Metastasis in 7–15 regional lymph nodes, N3b. Metastasis in 16 or more regional lymph nodes, M0. No distant metastasis, M1. Distant metastasis. Modified from (Brierley JD, Gospodarowicz MK, 2017).

Table 3. Patient and tumors characteristics included in the studies I–III.

Number of patients	All. N (%)	Intestinal. N (%)	Diffuse. N (%)
All	244	190 (77.9)	54 (22.1)
Median age at diagnosis (range)	72.3 (32.9-90.9)	74.4 (32.9–90.9)	66.8 (36.9–85.1)
Patient sex			
Female	101 (41.4)	68 (35.8)	33 (61.1)
Male	143 (58.6)	122 (64.2)	21 (38.9)
Site of primary Tumor			
Distal esophagus	19 (7.8)	19 (10.0)	
[a] GOJ/Cardia	60 (24.6)	60 (31.6)	
Corpus	106 (43.4)	52 (27.4)	
Antrum/pylorus	59 (24.2)	59 (31.1)	
Tumor differentiation (Grade)			
Grade I	17 (7.0)	17 (8.9)	0 (0)
Grade II	93 (38.1)	93 (48.9)	0 (0)
Grade III	134 (54.9)	80 (42.1)	54 (100.0)
[b] Stage			
I	46 (18.9)	40 (21.1)	6 (11.1)
II	102 (41.8)	79 (41.6)	23 (42.6)
III	83 (34.0)	61 (32.1)	22 (40.7)
IV	13 (5.3)	10 (5.3)	3 (5.6)
Follow-up status			
Alive and free of disease	48 (19.7)	34 (17.9)	14 (25.9)
Alive with disease	1 (0.4)	1 (0.5)	0 (0)

[a] GOJ, gastroesophageal junction.
[b] Staging according to the WHO classification manual for studies I–II (Bosman et al.,2010), while tumor staging was assessed according to the current TNM classification manual for study III (Brierley JD, Gospodarowicz MK, 2017).

4.2 Methods (I–III)

4.2.1 Tissue microarray construction (I–III)

The next-generation tissue microarray (ngTMA) technique was used for the tissue microarray constructions (Zlobec et al., 2014). Next-generation tissue microarrays (ngTMA) combine histological knowledge with digital pathology and automated tissue microarray instead of the conventional TMA. The TMA construction is difficult, time-consuming, and indistinct.

The representative paraffin blocks with tumor tissue were initially selected from each case; the blocks were selected by assessing the archived original hematoxylin-eosin (H&E) stained sections, then, new H&E slides were constructed, scanned by (Panoramic P250, 3DHistech), and saved into the university portal (case center.utu.fi). The analysis of the digital slides was done by using the Viewer software (3DHistech). Two areas were selected from the center of the tumor tissue and two spots from the invasive front of the tumor tissue using the 1.0 mm annotation tool, and different annotation colors were given to each part. Lastly, each corresponding tumor tissue core from the annotated slides was transferred to the TMA blocks with the automated TMA machinery (TMA Grandmaster, 3DHistech). One core representing the adjacent non-neoplastic mucosal tissue was selected from each tumor to serve as a control. The constructed TMA blocks were sectioned, stained, scanned, and images were uploaded into the web portal. The different steps of ngTMA constructions together with immunohistochemistry and in situ hybridizations samples are presented in Figure 8.

4.2.2 Immunohistochemistry and in situ hybridization (I–III)

The samples included in the studies I-III were from the same TMAs. Immunohistochemistry reactions were performed on 4-μm sections of each tumor on the TMA slides with BenchMark XT (Ventana/Roche) with UltraView Universal Diaminobenzoidin (DAB) detection kit. All primary antibodies used in study I–III with staining procedures are described in Table 4.

The antibodies used in study 1 were MHL1, MSH2, MSH6, PMS2, TP53, and E-cadherin. The tumor was classified as MMR-D when the complete loss of nuclear reactivity of one or more of the mismatch repair protein markers (MLH1, MSH2, MSH6, or PMS2) in tumor cells. At the same time, MMR-P status was considered when the tumor cells showed a positive nuclear reaction of at least one of the markers (MLH1, MSH2, MSH6, and PMS2). The positive expression of any of the antibodies used in the adjacent nonneoplastic epithelium, lymphocytes, stromal, and smooth

muscle cells was considered positive internal controls. The TP53 tumors were considered aberrant if there was a complete loss / or strong diffuse nuclear positivity of P53 expressions in the tumor cells (Köbel et al., 2016). Tumors with a moderate or weak nuclear reaction of P53 staining were classified as wild-type tumors. Tumors were-assessed as E-cadherin aberrant if a complete loss or only weak cytoplasmic response of the membranous reactivity of E-cadherin staining was detected in the tumor cells; in the other hand, tumor cells with a moderate or vigorous- membranous reaction of E-cadherin was scored as wild-type.

The antibodies included in study II are CD3, CD8, and FOXP3. The protocol used for CD3 and CD8 staining was the mild (30 min) protocol, with an antibody incubation time of 28 min at 37°C for CD3 staining and 32min at 37°C for CD8 staining. The epitope retrieval was performed with CC1 buffer (Ventana/Roche), and the ultra-view amplification kit (Ventana/Roche) was used with 4-min incubation. For FOXP3, staining was performed according to the manufacturer's instructions. The CD3+, CD8+ T lymphocytes were counted if the positive membranous reaction was detected in the lymphocytes in the tumor's central and invasive fronts. FOXP3 was evaluated as positive when there was a positive nuclear reaction of lymphocytes in the central part and the invasive front of the tumor tissue. Invasive fronts and central parts of the tumor tissue were evaluated separately.

In study III, for FHOD1 and FMNL1 immunohistochemical staining of tissue sections, the peroxidase method was performed using a LabVision autostainer device (LabVision/Thermo Fisher Scientific, Cheshire, UK). The slides were first treated with citrate buffer for antigen retrieval, then 3% H2O2 was added for 10 min to suppress the endogenous peroxidase. The slides were then incubated with normal non-immune serum at 37°C for 30 min and then incubated with affinity-purified rabbit-anti FHOD1 and FMNL1 antibodies at 4°C for 60 min (Table 4). After that, slides were washed with PBS, then incubated with BrightVision Poly-HRPAnti-mouse/rabbit rat IgG (Immunologic, Duiven, Netherlands Duiven, Netherlands) for 30 min, followed by3-3'-diaminobenzidine (Dako, Glostrup, Denmark) for 10 min, then the reaction was stopped by a rinse with PBS, then the slides were counteracted with Hematoxylin. The expression of FMNL1 and FHOD1 staining was cytoplasmic and was scored as negative (0), mild (1), moderate (2), or strong (3). Endothelial cell, stromal, and muscle cell expressions were regarded as internal positive controls.

4.2.3 In situ hybridization (I)

For EBV-status detection, a ready-to-use EBV-encoded small RNA probe (EBER) (Ventana/Roche) was used together with ISH-Protease 3 pretreatment for 28 min and 1-h probe incubation, signals detected by the ISH iVIEW Blue Detection Kit. Tumors were classified as positive for EBV if EBER in situ hybridization analysis

showed a positive nuclear reaction. On the other hand, if no nuclear response was detected, tumors were classified as negative for EBV infection.

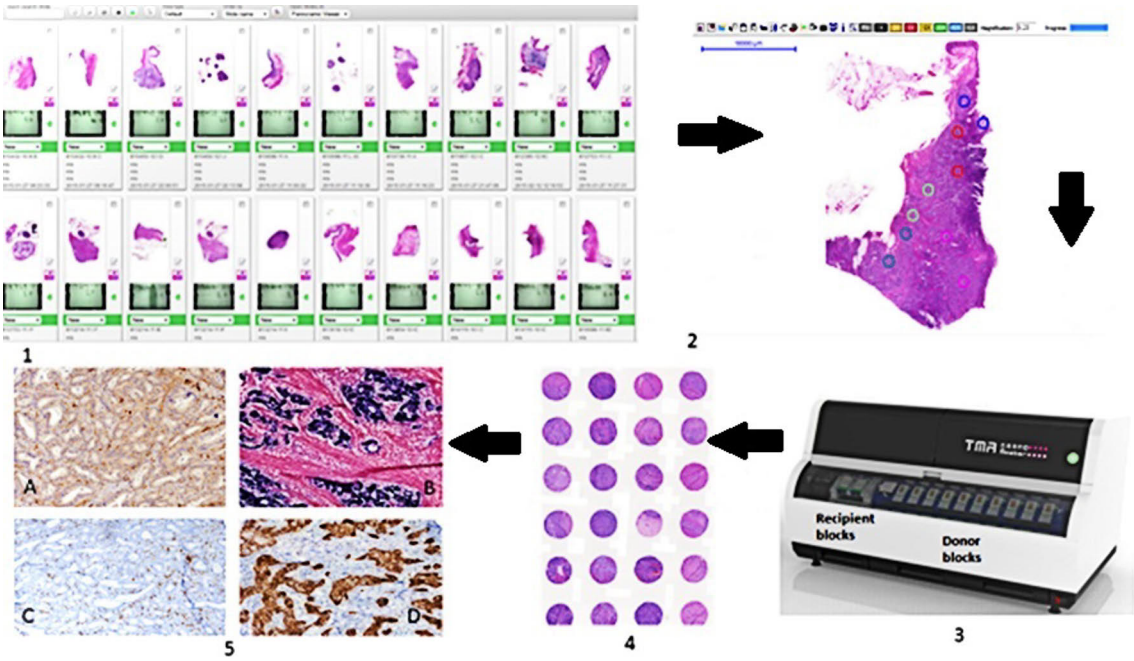

Figure 9. Tissue microarrays (TMA) constructions and immunohistochemistry (IHC). The process of the TMA construction started with the selection and scanning of tissue specimen into a web portal (1); then, multiple annotations of tumor tissue were done, the annotation colors indicating central part, invasive front, and adjacent mucosa (2). The construction of recipient blocks from donor blocks was done by the TMA Grandmaster Machine(3), Then the TMA was stained first by H&E staining (4); after that, the new TMAs blocks were stained by IHC and utilized for the in situ hybridization to detect the EBER1. Examples of IHC and in situ hybridization staining (5): FHOD1 + (A), in situ hybridization EBER+ (B), CD3+ (C), and E-cadherin wild type(D).

4.2.4 Gastric cancer cell lines, Western blot analysis, and immunofluorescence staining (III)

4.2.4.1 Gastric cancer cell lines

Gastric cancer cell lines used in study III were AGS, MKN28, and MKN45. All cell lines were cultured in DMEM (Lonza, Basel, Switzerland) containing 10% fetal bovine serum (Biowest, Nuaillé, France) and supplemented with 5 mM ultra glutamine and 100 U/ml penicillin-streptomycin (Gibco, CA, USA). The AGS cell line was derived from the primary gastric cancer of a 54-year-old female and represented mixed diffuse and intestinal types. The MKN28 cell line was derived from a 70-year-

old female with a lymph node metastasis of a well-differentiated primary gastric cancer of intestinal histology. The MKN45 cell line was derived from a liver metastasis of a 62-year-old patient with poorly differentiated primary gastric cancer of diffuse histology (Barranco et al., 1983; Motoyama et al., 1986). All cell lines used were publicly available.

4.2.4.2 Western blot analysis

Cells were harvested and lysed in RIPA buffer solution supplemented with inhibitors. The insoluble cell debris was removed by centrifugation, and the samples were normalized for protein concentration by the Bradford method (Bio-Rad, after that, a Laemmle buffer was added to the samples). The total protein was separated equally by SDS-PAGE and transferred to the nitrocellulose membrane. The membranes were blocked with 5% dry milk, then immunoblotted with different antibodies diluted in 5% bovine serum albumin in TBST (Tris-Buffered Saline Tween-20); the rabbit anti-human FHOD1 or FMNL1 (1:1000, Sigma-Aldrich, St. Louis, MO). Antibodies were incubated overnight at 4°C. HRP conjugated rabbit polyclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000, Abcam, Cambridge, UK) was used as a control for protein loading followed by a secondary antibody, HRP-conjugated swine anti-rabbit, and HRP-conjugated rabbit anti-mouse IgG (1:3000, Dako, Glostrup, Denmark). Membranes were washed three times with TBST between the different steps. Bound proteins were detected by enhanced chemiluminescence.

4.2.4.3 Cell immunofluorescence staining and microscopy

Cells were plated on gelatin (Sigma-Aldrich) pre-coated coverslips (13 mm) and grown in complete medium for 24 hours. The cells were fixed with 4% paraformaldehyde for 10 min at room temperature. After washing the coverslip with PBS, they were blocked with 3% BSA, 5% dry milk, and 0.5% Triton X-100. Primary rabbit anti-human FHOD1 or FMNL1 antibodies (1:200, Sigma-Aldrich) were incubated for 1 hour at room temperature. Next, the coverslips were incubated with the secondary antibody Alexa Fluor 568 goat anti-rabbit IgG (1:500, Invitrogen, Carlsbad, CA). For negative controls, coverslips were stained using secondary and phalloidin antibodies only, the filamentous actin was visualized with Alexa Fluor 488-conjugated phalloidin (1:500, Invitrogen). The mounting media contained DAPI for staining nuclei (ProLong Gold Antifade Mountant with DAPI, Life Technologies). Images were taken with a Nikon Elipse Ni fluorescence microscope (Nikon Instruments).

Table 4. Primary antibodies and dilutions are used in immunocytochemistry (IHC), in situ hybridizations (ISH), and Western blotting (WB).

IHC

Study	Antibody/ Probe	Clones	Dilution	Reagents, signal detection	Antibody/ incubation	Provider
I	MLH1	G168-15	1:5	Ultra View Universal DAB* Detection Kit, Bench Mark XT(Ventana/Roche) &amplification kit	36 min	BD.Pharminogen
	MSH6	EP49	1:200	”	32 min	
	MSH2	G219-1129	”	”	28 min	BD.Pharminogen
	PMS2	EPR3947	Ready-to-use	Opt iView Universal DAB Detection Kit & amplification kit(Ventana/Roche)	44min	Ventana/Roche
	TP53	Bp53-11	”	Ultra View Universal DAB Detection Kit, BenchMark XT (Ventana/Roche)	28 min	Ventana/Roche
	E-cadherin	NHC-38	1:100	Ultra View Universal DAB Detection Kit, BenchMark XT(Ventana/Roche)& amplification kit	32 min	Agilent Technologies
II	CD3	2GV6	Ready-to-use	Ultra View Universal DAB Detection Kit, BenchMark XT (Ventana/Roche)	28 min	Ventana/Roche
	CD8	SP57	”	UltraView Universal DAB Detection Kit, BenchMark XT (Ventana/Roche)	28 min	Ventana/Roche
	FOXP3	236A/E7	1:200	UltraView Universal DAB Detection Kit, Bench Mark XT (Ventana/Roche)&amplificatio n kit	30 min	Abcam
III	FHOD1	HPA008129	1:150	Streptavidin-peroxidase method.LabVision Thermo Fisher. BrightVision Poly-HRPAnti-mouse/rabbit rat IgG (Immunologic, Duiven, Netherlands) detection kit	60 min	Sigma-Aldrich Corporation, St Louis, MO, USA
	FMNL1	HPA024468	1:500	”	60 min	“

ISH

Study	Target Gene	ISH probe	Reagents, signal detection	Antibody/ incubation	Provider
	EBV	EBER (Epstein-Barr virus-encoded small RNA	ISH iView Blue Detection Kit, BenchMark XT (Ventana/Roche)	60 min	Ventana/Roche

WB

Study	Primary antibody	Clones	Dilution	Incubation	Provider
	FHOD1	HPA008129	1:1000		Sigma/Aldrich
	FMNL1	HPA024468	1:1000		Sigma/Aldrich
	GAPDH**	Ab8245	1:5000 1:3000	Overnight 60 min	Abcam, Cambridge, UK Dako, Glostrup, Denmark

*DAB, 3-3`-diaminbezodinine . **GADPH, glyceraldehyde-3-phosphate dehydrogenase

4.3 Ethical considerations

All studies were conducted following the declaration of Helsinki and the Finnish legislation for the use of archived tissue samples and associated clinical information. The relevant clinical data were regained, and the histological samples were gathered and analyzed under the approval of the National Authority for Medico-Legal Affairs. The Institutional Review Board of the Hospital District of Southwest Finland and Auria Biobank's permission to host the specimen archive (project number AB14-5616). Biobank act provides access to human samples and associated clinical information gathered during clinical/diagnostic procedures after the biobank's scientific-ethical board review. Therefore, informed permission from surviving patients was not required.

4.4 Statistical analysis (I–III)

Statistical analyses were performed with IBM SPSS Statistics for Windows (IBM Corporation, Armonk, NY), version 21.0 for study I, version 24.0 for study II, and, in study III, statistical analyses were performed with IBM SPSS Statistics for Windows, version 27.0 (IBM Corporation, Armonk, NY). The association between the IHC results and the clinicopathological variables was analyzed with the $\chi 2$ test or Fisher's exact test for discrete variables and one-way ANOVA for continuous variables. Two × two tables were used to calculate odds ratios (OR) and 95% confidence intervals (CI) using the exact method. The Kaplan-Meier method, log-rank test, and Cox's proportional hazards regression model were used for univariate survival analysis. Multivariate survival analysis was performed by Cox's proportional hazards regression model. Recurrence-free survival (RFS) was calculated from diagnosis to the time of first recurrence, death of any cause, or the last follow-up date. Overall survival (OS) was calculated from diagnosis to death of any cause or the last follow-up date. The Student's T-test was used to calculate mean and median P values for normal distribution values, while the Mann-Whitney U test was used for non-normal distribution values. The methodology of statistical analyses has been presented in detail in the original publication (I–II).

5 Results

5.1 The frequency of MMR-D, TP53, E-cadherin immunohistochemistry expression, and EBV in situ hybridization in intestinal and diffuse-type GC (I)

The study cohort consists of a consecutive series of 244 patients diagnosed with adenocarcinoma of the stomach, the gastro-esophageal junction (GOJ), or distal esophagus at the Turku University Hospital between years 1993 and 2012. Starting with Laurén's classification, 190 tumors were the intestinal type, and 54 were the diffuse type used as control. EBV, MMR, and TP53 status could be analyzed from 238 tumors, and E-cadherin was evaluated in 234 tumors. Due to inadequate tissue material, the markers could not be evaluated in the remaining tumors. Of the 186 intestinal tumors available for IHC and in situ hybridization, EBV RNA was detected in 17 (9.1%). MMR-D was detected in 19 (10.2%) cases, while none of the diffuse tumors (N=50) had MMR-D phenotype or were EBV RNA positive tumors (Fisher´s exact test, p= 0.017). In addition, intestinal-type tumors showed more common aberrant TP53 expression than diffuse-type tumors (55.4% vs. 19.2%, Fisher´s exact test, p <0.0001).

In addition, ninety-four cases (39.5%) were found to show a combination of EBV negativity, MMR-P, and TP53 wt (this subtype will refer to as the "other" subtype throughout this work). Among the "other" subtype, 52/186 (28.0%) tumors were of intestinal-histology, while 42/52 (80.8%) were of the diffuse-histology tumors ($p < 0.0001$). Aberrant E-cadherin expression could be detected in 3/183 (1.6%) among the intestinal-type tumors, whereas aberrant E-cadherin expression could be seen in 25/49(51.0) diffuse-type GC. Among the "other" tumors, *i.e.*, the EBV negative, MMR-P, and TP53 wild-type tumors, 21/39 (53.8%) of the diffuse-type GCs but none of the intestinal-type GCs ($n = 51$) had aberrant E-cadherin expression.

Furthermore, none of the MMR-D tumors was EBV positive and TP53 aberrant. Thus, in study I, we started with Lauren's classification, and according to EBV pos-

itivity, MMR-D status, and TP53 expression, we could detect four different gastroesophageal cancers, including EBV+ , MMR-D, TP53, and "other" subtypes. The frequency of the GC subtypes and anatomical location are presented in Figure 10.

5.2 TP53, EBV, and MMR- status concerning clinicopathological variables and survival (I)

Among the intestinal-type GCs, we examined the correlation between the EBV, MMR-D, and TP53 status and the clinical variables, including anatomical site, gender, tumor grade, and stage of the disease. Our results showed that aberrant TP53 expression was more common in proximal than distal tumors ($p = 0.002$). In contrast, tumors with MMR-D were mainly located distally ($p = 0.042$). In addition, MMR-D tumors were more common among females than males ($p = 0.042$), whereas EBV+ tumors were more common among males than female patients ($p = 0.035$). Interestingly, diffuse-type GC tumors are usually considered poorly differentiated tumors; however, among the intestinal type tumors, the EBV+ tumors were often of high grade ($p < 0.0001$) and primarily located in the gastric corpus ($p = 0.011$). No significant associations could be detected between the "other" subtype (MMR-P, EBV- and TP53 wt subtype) with any examined clinicopathological variables; this could be because of a small sample of diffuse GC in our cohort.

We detected a significant association between the patient's age (below or above the median, the median was 72.3 years), size of the tumor (T1-T2 versus T3-T4), stage of the disease (stage-II versus stage III-IV), and the MMR-D status with OS. MMR-D status was predictive for prolonged OS in both univariate ($p = 0.040$) and multivariate ($p = 0.015$) analysis; however, the MMR-D status was not associated with recurrence-free survival (RFS). In intestinal-type tumors, increasing depth of tumor invasion was associated with shorter RFS and OS (RFS, $p = 0.045$; OS, $p = 0.031$). Similarly, increasing tumor stage was associated with shorter RFS and OS (RFS, $p = 0.020$; OS, $p < 0.0001$). Shorter RFS and OS were associated with patient age above the median at the diagnosis time. (RFS, $p = 0.006$; OS, $p = 0.026$). No significant associations were observed between TP53 or EBV status and survival. Table 7 presents the association of EBV positive, MMR-D status, and TP53 with selected clinical variables

Table 5. The association between some molecular markers and selected clinicopathological variations in intestinal-type gastric cancer tumors. The figures show the number of patients (%). Significant p values are indicated in bold.*

	EBV positivity	*P* value	MMR-D	*P* value	TP53 aberration	*P* value
Patient sex		**0.035**		**0.042**		NS
Female	2 (11.8)		11 (57.9)		34 (33.0)	
Male	15 (88.2)		8 (42.1)		69 (67.0)	
Location		**0.011**		**0.002**		**0.010**
Distal esophagus	0 (0)		0 (0)		15 (14.6)	
GOJ/Cardia	7 (41.2)		2 (10.5)		39 (37.9)	
Corpus	9 (52.9)		4 (21.1)		23 (22.3)	
Antrum-Pylorus	1 (5.9)		13 (68.4)		26 (25.2)	
Grade		**<0.0001**		NS		NS
I	0 (0)		1 (5.3)		10 (9.7)	
II	2 (11.8)		8 (42.1)		54 (52.4)	
III	15 (88.2)		110 (52.6)		39 (37.9)	
Tumor size (T)		NS		NS		NS
T1-2	5 (29.4)		3 (15.8)		28 (27.2)	
T3-4	12 (70.6)		16 (84.2)		75 (72.8)	
Stage		0.768		0.624		
I–II	11 (64.7)		13 (68.4)		65 (63.1)	
III–IV	6 (35.3)		6 (31.6)		38 (36.9)	

EBV = Epstein-Barr virus, GOJ = gastroesophageal junction, MMR-D = mismatch repair protein -deficient.

NS = not specific.

*The comparison groups presented in this table are only for EBV+, MMR-D, and TP53 and the selected clinical parameters. Detailed information is shown in the original publication I. Modified from the original publication I, page 8. Authors are the copyright holders.

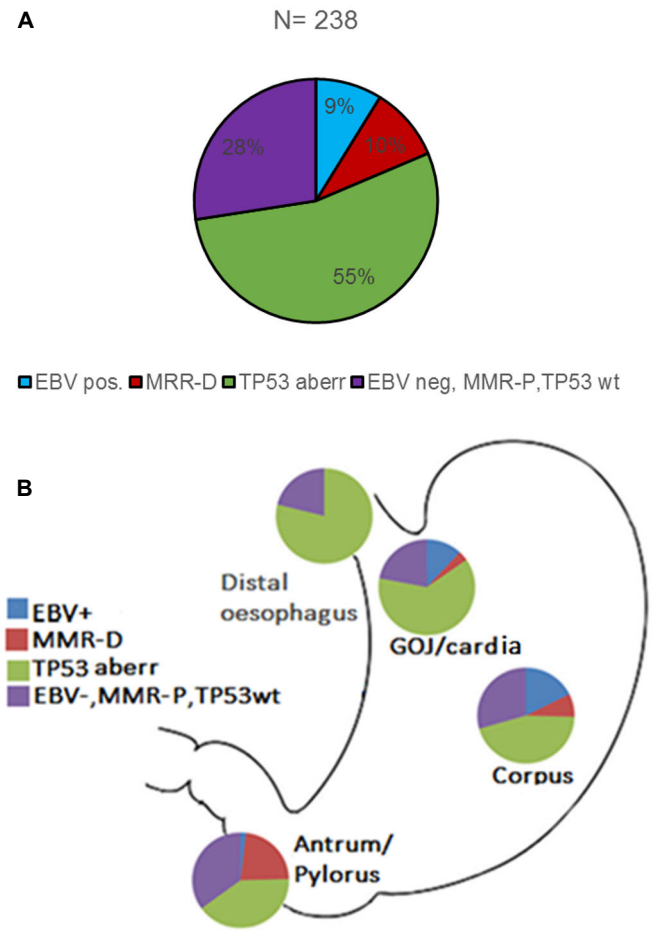

Figure 10.Frequency and anatomic distribution of EBV positive, MMR-D, TP53 aberrant, and" others" subtypes among intestinal adenocarcinomas of the stomach, gastro-oesophageal junction, and distal esophagus. The original classification was based on immunohistochemistry and in situ hybridization. A. Frequency of the four molecular subtypes of intestinal-type oesophagogastric cancer. B. Distribution of the four molecular subtypes of intestinal-type oesophagogastric cancer in different anatomical locations.EBV Epstein-Barr virus GOJ gastro-oesophageal junction, MMR-D = mismatch repair protein-deficit, MMR-P = mismatch repair protein-proficient, wt. = wild type, aberr = aberrant. Modified from the original publication I, p.337. Authors are the copyright holders.

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5.3 The distribution of CD3+, CD8+, and FOXP3+ T lymphocytes among intestinal-type gastric cancer molecular subtypes (II)

In study II, we analyzed the presence of T lymphocytes in ngTMAs of 190 intestinal-type gastric cancers and their relation with the different molecular subtypes and clinical characteristics. CD3+, CD8+, and FOXP3 T lymphocytes were identified by immunohistochemistry and scored separately. CD3 expression could be evaluated in 180 (98%), CD8 expression in 170 (92%), and FOXP3 in 173 (94%) tumors. We attempted to evaluate the number of T lymphocytes separately at the invasive front and the central part of the tumor; however, no statistical differences were detected; thus, the results were combined for final analysis.

Our results showed that the number of T lymphocyte subsets varied among the different molecular subtypes. The EBV+ tumors had the highest absolute numbers of CD8+ ($p = 0.001$), CD3+ ($p = 0.002$) and FOXP3+ T lymphocytes ($p = 0.002$). In addition, EBV+ tumors showed the highest CD8+/FOXP3+ ratio ($p = 0.002$). No significant differences were detected in the number of intratumoral T lymphocytes between the three other molecular subtypes, *i.e.,* TP53 aberrant, MMR-D, and "other" subtypes.

Interestingly, the TP53 aberrant subtype contained low numbers of CD8+ and FOXP3+ lymphocytes compared to the TP53 wild type. In addition, TP53 aberration was associated with low CD8+/FOXP3+ ($p < 0.0001$) and CD3+/FOXP3+ ($p = 0.033$) ratios, suggesting an immunosuppressive condition in the TP53 subtype. Unexpectedly, even though there was a tendency for a high number of CD8+ cells in the MMR-D tumors, they did not statistically differ from TP53 aberrant or "other" (EBVneg, MMR-P, and TP53 wt) GC subtypes ($p = 0.096$). The distributions and the average number of CD8+, CD3+, and FOXP3+ T lymphocytes among each intestinal-type gastric cancer subtype are presented in Figure 11.

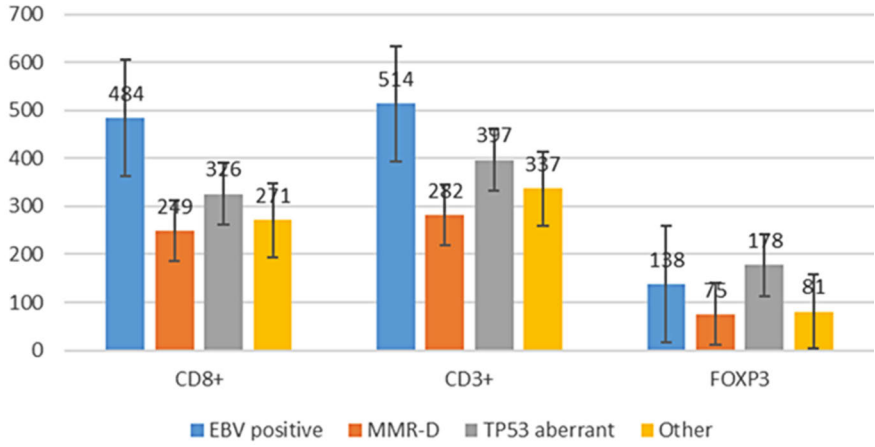

Figure 11. The average numbers and frequency of T lymphocytes among intestinal-type molecular subtypes of GC. In this chart, the EBV+ GC subtype differs from the other GC subtypes and shows the highest average values of CD8+, CD3+, and FOXP3+ T lymphocytes.

5.4 The expression of CD3+, CD8+, and FOXP3+ T lymphocyte among intestinal-type gastric cancer molecular subtypes: correlation with clinicopathological characteristics and survival (II)

The clinical variables tested in association with the T lymphocyte states included the degree of the tumor differentiation, tumor site, and gender of the patients (Table 3, page 712, original publication. Study II). Poor tumor differentiation was significantly associated with high values of CD3+, CD8+ T lymphocytes ($p = 0.001$,

p = 0.005 respectively) and the high ratio of CD8+/FOXP3+ cells (p = 0.018). Moreover, we detected low numbers of FOXP3+ T lymphocytes in the proximally located tumors (p = 0.029), whereas the high numbers of FOXP3+ T lymphocytes were associated with the male gender (p = 0.025). We did not detect any significant association between the number of intratumoral T lymphocytes and the following clinical variables: patient's age, tumor size, or tumor location.

Our results showed that the number of CD3+, CD8+, and FOXP3+ T lymphocytes correlated with the prognosis in patients with intestinal-type gastric cancer. In the univariate analysis, we found that high values of CD3+ cells were associated with longer RFS (HR 0.58, 95% CI: 0.35–0.97) and OS (HR 0.61, 95% CI 0.38–0.98), whereas the high amount of CD8+ cells was associated with OS (HR 0.59, 95% CI: 0.37–0.95). In multivariate analysis, high values of CD3+ T lymphocytes remain an independent prognostic factor for more prolonged RFS and OS and a high amount of CD8+ cells for OS (HR 17.2, 95% CI: 1.78–1660.0). (Figure 2, page 713, original publication. Study II). Another clinical parameter affecting the OS in patients with intestinal gastric cancer was the MMR status of the tumor. As expected, patients with MMR-D tumors had longer OS than patients with MMR-P tumors. Furthermore, we found that tumor size and advanced stage significantly affect the prognosis of intestinal-type gastric cancer. Larger tumor size, the more advanced stage of the disease correlates with worse RFS and OS; moreover, the advanced stage remained an independent prognostic factor for OS (HR 8.25, 95% CI: 2.40–28.3).

5.5 Expression pattern and localization of FHOD1 and FMNL1 formin proteins in gastric cancer cell lines (III)

The expression of FHOD1 and FMNL1 formin proteins within the molecular subtypes of intestinal-gastric cancer was investigated in study III. The expression and subcellular distribution of FHOD1 and FMNL1 formin proteins was studied in three publicly available gastric cancer cell lines, MK28, MK26, and AGS, representing the various degree of differentiation and morphological type of GC. All studied cell lines exhibited equal moderate to high FHOD1 and low FMNL1 expressions by western blot analysis. In addition, the localization and expression pattern was studied by double immunofluorescence staining of F-actin and FHOD1 or FMNL1 in the same cell lines. The distribution of FMNL1 was cytoplasmic in all studied cell lines, while FHOD1 expression localized both in the cytoplasm and along the actin filaments in a dotted fashion. Western blotting and immunofluorescent staining results are illustrated in Figure 2, original publication, study III.

5.6 Characterization of FHOD1 and FMNL1 in the non-neoplastic gastric mucosal lining and gastric cancer clinical samples (III)

The expression pattern, intensity, and localization of FHOD1 and FMNL1 formins were analyzed in clinical FFPE tissue samples and the non-neoplastic adjacent mucosa. The mucosa adjacent to tumor tissue showed no or mild expression of FHOD1 or FMNL1 formin protein. Out of 244 gastric cancer tissues examined, the expressions of FMNL1 could be assessed in 234 (99.5%) cases, while FHOD1 could be evaluated in 227 (93%) cases. The rest of the cases were not evaluated because of insufficient tissue material for IHC assessment. Our results indicated that FMNL1 and FHOD1 could be detected in the cytoplasm of tumor cells. The staining intensity was scored from low to high: score 0 = no staining, 1 = weak , 2= intermediate intensity, and score 3 = intense staining. Our results indicated that FHOD1 and FMNL1 were upregulated during cancer development and could-affect tumor progression. Attempts were made to evaluate the peripheral and central parts of the tumor separately; however, results showed no significant statistical difference. The number of tumors with differential formin expression intensity in the central and peripheral parts of the tumor samples is summarized in Table 6.

Table 6. FMNL1 and FHOD1 Immunohistochemical expressions according to the most intense staining, central and peripheral parts of the tumor were assessed separately. The values represent the % (N=244).

FMNL1	**FMNL1 CENTRAL N (%)**	**FMNL1 PERIPHERAL N (%)**	**FMNL1 TOTAL N (%)**
Negative + Weak	115 (47)	65 (27)	73 (30)
Moderate + Strong	106 (43.4)	139 (57)	161 (66)
Missed	23 (9.4)	40 (16.4)	10 (4.1)
FHOD1	**FHOD1 central N (%)**	**FHOD1 peripheral N (%)**	**FHOD1 total N (%)**
Negative + Weak	35 (14.3)	214 (87.3)	14 (5.7)
Moderate + Strong	174 (71.3%)	14 (5.7)	213 (87.3)
Missed	35 (14.3%)	17 (7.6)	17 (7.0)

5.7 Combined results of FMNL1 and FHOD1 expression with values of T lymphocyte infiltration in intestinal gastric cancer clinical samples (III)

We combined the evaluation results of intratumoral T lymphocytes infiltration with FHOD1 and FMNL1 expression from the same tumors to assess whether an association exists between the tumoral expression FHOD1 and FMNL1 and intratumoral CD3+, CD8+, and FOXP3+ T lymphocytes. We found tumors with moderate FHOD1 staining intensity in the tumor cells harbored significantly higher values of CD8 + cells, both in the central and peripheral parts of the tumor ($p = 0.039$ and $p = 0.003$, respectively), indicating a possible immunogenic trigger of the up-regulation of FHOD1 in intestinal-type GC. Similarly, tumors with high FHOD1 expression in the tumor cells were associated with higher CD3+ T lymphocytes in the central part of tumor samples; however, this association was not statistically significant ($p = 0.090$). On the other hand, FMNL1 expression in the tumor cells did not correlate with intratumoral T lymphocyte infiltration indicating an indirect effect of tumor FMNL1 upregulation on the intratumoral immune response (Table 3, original publication. Study III).

5.8 The expression of FHOD1 and FMNL1 in relation to clinical variables among intestinal-type gastric cancer molecular subtypes and survival (III)

We studied the association between selected clinic-pathological variables and the expression of FHOD1 and FMNL1 in GC clinical samples. The variables were age and sex, tumor stage, tumor location, tumor size, and molecular tumor subtype (TP53, MMR status, and EBV). Our results showed a significant association between the moderate expression of FMNL1 in the peripheral part of the tumor and increased tumor size and more advanced stage of the disease (stage II and stage IV). This finding suggests that FMNL1 is elevated upon tumor progression

Interestingly, our results showed a statistically significant association between FHOD1 expression and the GC molecular subtype with aberrant TP53, tumors with negative FHOD1 expression in their center part were significantly connected with mutated TP53. On the other hand, FMNL1 expression was not significantly connected to any molecular subtypes. These results are presented in Table 2, original publication study III.

In addition, study III assessed the prognostic value of FHOD1 and FMNL1 mRNA expression. We utilized the available km-plotter database for Kaplan-Meier survival analyses (Szász et al., 2016). In this analysis, FHOD1 and FMNL1 mRNA

expression was associated with reduced OS in intestinal-type- GC patients (Figure 12). However, the elevated expression of both FHOD1 and FMNL1 in the clinical samples of intestinal-type GC did not show any association with disease outcome (OS or RFS), our result indicating FHOD1 and FMNL1 are not prognostic indicators in intestinal-type molecular subtypes GC.

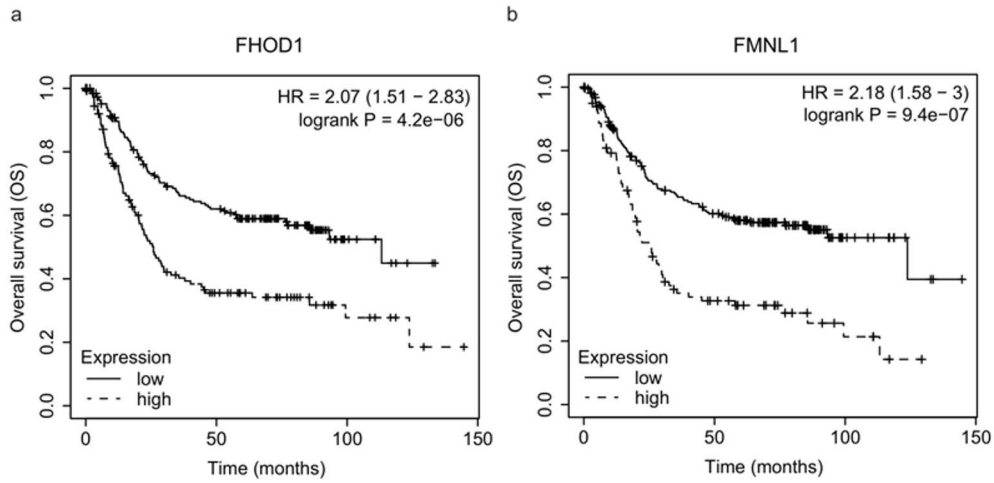

Figure 12. Correlation between FHOD1 and FMNL1 mRNA expression and overall survival in gastric cancer-intestinal type samples. A. Kaplan–Meier analysis (OS) of FHOD1 mRNA expression. B. Kaplan–Meier analysis (OS) of FMNL1 mRNA expression. Modified from the original publication III, p 4. Authors are the copyright holders.

6 Discussion

6.1 Molecular subtypes of gastric adenocarcinoma (I)

Recent molecular studies, The Cancer Genome Atlas (TCGA) and Asian Cancer Research Group (ACRG), have classified gastric cancer into molecular subtypes. These studies demonstrate that gastric cancer is not a single disease (Bass et al., 2014; Cristescu et al., 2015). The variable molecular background in gastric cancer is reflected in the behavior of the different molecular subtypes; accordingly, in attempts to develop optimal targeted treatment, we need robust methods for diagnosing the GC molecular subtypes. Furthermore, the required technique should be easy and applicable in routine pathology practice. In study I, we utilize the findings from the previous molecular classification of gastric adenocarcinomas introduced by the TCGA and ACRG to test the suitability of the proposed characterization algorithm for personalized treatment and clinical diagnostics by using simple methods. We could detect four subgroups of GC with distinguished molecular and clinical characteristics using accurate and straightforward techniques. Our analysis is based on the Laurén classification, immunohistochemistry, and *in situ* hybridization. These methods may be helpful for clinical, diagnostics, and research purposes.

Other research groups have attempted a similar practice to our approach (Ahn et al., 2017; Bass et al., 2014; Cristescu et al., 2015; Díaz Del Arco et al., 2018; Huang et al., 2019; Kim et al., 2016b; Setia et al., 2016); each of these research groups has used some diversity of the original work by the TCGA consortium, and ACGR. In contrast to the previously mentioned publications, in our analysis, we used a subtyping algorithm that combines some aspects of TCGA and ACRG, starting with the Laurén classification to first divide gastric cancer into two groups intestinal and diffuse gastric cancers and then to identify the subtypes with IHC and ISH methods.

In study I, we investigated tumors of the distal esophagus, GOJ, and cardia as a single group of proximally located tumors because the distinction criteria between oesophageal, gastro-oesophageal, and proximally located gastric carcinomas are controversial. According to The Cancer Genom Atlas Working Group (2017), this grouping appears justified, as the molecular signature of esophagus adenocarcinoma has shown genetic similarity to the CIN GC subtype.

Moreover, the focus of our analyses, intestinal-type GC, is known to present more diverse molecular profiles than diffuse-type tumors. A small subset of diffuse-type GC was included in our cohort to serve as a reference group. Therefore, the percentages for different markers for diffuse-type tumors should be considered approximate. Diffuse-type GC was observed to be predominantly a genomic-stable subtype according to TCGA (Bass et al., 2014) or tumors with MSS/EMT features according to ACRG (Cristescu et al., 2015). In our analysis (study I), all tumors with aberrant E-cadherin expression were classified as diffuse-type according to the Laurén classification. The proportion of intestinal-type tumors with aberrant E-cadherin is comparable to the frequency of E-cadherin mutations (4.1%) detected in the study by the TCGA consortium (Bass et al., 2014). Only three intestinal-type tumors with aberrant E-cadherin expressions were detected in our sample set, and they were shown to belong to either EBV+, MMR-D, or TP53 aberration subtypes.

Among intestinal-type GC tumors, the frequency of EBV+ GC subtype was 9.1%, which is in line with the results of 3.0–9.5% from other studies (Bass et al., 2014; Cristescu et al., 2015). The frequency of MMR-D subtype tumors constituted 10.2% in our cohort, which is comparable to the result of 9.2% obtained by Kim et al. (2016) but somewhat less than 24.5–26.0% found in other studies (Bass et al., 2014; Cristescu et al., 2015). The discrepancies could be due to methodology differentiation or geographical variations among the different cohorts.

Aberrant TP53 expression frequency was 55.4% in our cohort, close to the 53.1% of the TCGA study but slightly different from 31.3% of ACRG, or 67.3% of Kim et al.(2016). According to Global Cancer Statistics (2020), the incidence of GC showed a significant geographic discrepancy between Asia and Europe; in addition, *TP53* mutations are widespread (around 50% in primary tumors and up to 70% in metastatic) among GC. Thus, the frequency of TP53 aberrant GC subtype differences between European and Asian cohorts are expected due to geographical, genetic, or environmental factors (Sung et al., 2021).

Our results showed intestinal-type EBV + and MMR-D GC subtypes. These two subtypes were exclusive with no overlap; this result was consistent with other studies (Bass et al., 2014; Puliga et al., 2021). None of the EBV neg, MMR-P, and TP53 wt intestinal-type tumor subtypes (the "other" subtype) showed aberrant E-cadherin expression, and on the other hand, none of the diffuse-type tumors were found to be EBV+ or MMR-D. These observations imply that using Laurén classification followed by EBER in situ hybridization and MMR protein detection by the IHC could be sufficient for general tumor characterization without the need to perform E-cadherin or even TP53 staining. Recently, Huang et al. (2019) have applied a similar strategy. In the Hung et al. study, the researchers categorized the EBV+ and MMR-D GC subtypes utilizing the molecular classification by the TCGA and the traditional histological category, the Laurén system. Initially, the authors classified the EBV+

and MMR-D GC subtypes; then, they divided the remaining tumors histologically into intestinal- and diffuse-type. However, an additional biomarker could be needed to characterize further a subgroup of intestinal-type tumors that show neither EBV +, MMR-D, nor TP53 or E-cadherin aberration (Huang et al., 2019).

Opposite to our study, some studies showed that a small proportion of diffuse-type tumors are either EBV+ or MMR-D (Ahn et al., 2017; Bass et al., 2014; Cristescu et al., 2015; Kim et al., 2016). However, Cristescu et al. (2015) reported MMR-D in 17% of the diffuse-type tumors. The proportion of diffuse-type tumors with TP53 aberration in our study was quite similar to some other reports (Bass et al., 2014; Cristescu et al., 2015), but notably different from the 54% observed by (Kim et al., 2016). In addition to methodological variations and sample size, some differences in the proportions of various markers may result from molecular variation between tumors derived from ethnically diverse patients.

6.2 Prognostic implications of intratumoral T lymphocytes in correlation to molecular subtypes gastric cancer (II)

The introduction of modern immunotherapy has modified cancer treatment (Lazăr et al., 2018). Immunotherapies target the immune cells instead of tumor cells. In GC, the current immunotherapies are under investigation in clinical trials. The randomized phase 3 trial (KEYNOTE-061), pembrolizumab, which is FDA-approved immunotherapy, did not significantly improve the OS of patients with advanced or metastatic GC. However, pembrolizumab showed a better safety profile than paclitaxel chemotherapy. Further trials of pembrolizumab and the analysis of the tumor microenvironment in GC are still ongoing (Shitara et al., 2018). Studies on the connection between tumor microenvironment, mainly CALs and cancer cells among various solid tumors, including GC, provide valuable information relevant for prognostication and predictive diagnostics (Wang et al., 2017).

As the clinical significance of the GC molecular subtypes is unknown yet, studying the tumor immunity among the GC molecular subtypes is especially relevant (Bass et al., 2014; Wang et al., 2020; Apicella et al., 2017). Our results indicate that, among the molecular subtypes, EBV+ gastric cancers are the most susceptible to host immunity, and patients with EBV+ GC could be a candidate for immunotherapy and cancer-immune-vaccination. Others (Sunakawa & Lenz, 2015; Kang et al., 2016; Kwak et al., 2020) have also reported similar findings. Independent studies on the association between EBV+ GC and intratumoral lymphocytes have shown results generally in line with our findings (Cho et al., 2016; de Rosa et al., 2018; Kim et al., 2019 b). However, in contrast to these studies, our analysis focused on intestinal-

type gastric cancer because it is the histology-type GC with more molecular heterogeneity. Our work-study II provides more profound and comprehensive information about the distribution and amount of cancer-associated T lymphocytes in the four molecular GC subtypes.

Parallel studies analyzing the correlation of tumor-infiltrating T lymphocytes with the molecular GC subtypes have been published (de Rosa et al., 2018; Jiang et al., 2018; Junttila et al., 2020; Lee et al., 2018), with varying cohort sizes and the number of clinical parameters. Our analysis includes a relatively large sample set of intestinal gastric cancers of Caucasian origin and includes extensive longitudinal clinical information compared to these studies. Our study also included selected clinical parameters together with DFS and OS. Weaknesses of our study include results from a single institute and the fact that the cohort consists of patients from several decades, during which clinical practices and reporting may have changed.

In study II, our results showed that high CD8+ T lymphocytes were a robust prognostic indicator and associated with prolonged OS among the four molecular subtypes. Furthermore, increased numbers of CD3+ T lymphocytes were significantly associated with more prolonged RFS of the patients, which remained an independent prognostic factor for prolonged OS in multivariate analysis. This result was in line with the reported study by Lee et al., where CD3+ was an independent favorable prognostic factor in gastric cancer (Lee et al., 2008).

A novel and interesting finding in study II was the association of TP53 aberrant subtype with T lymphocyte infiltrate. While TP53 aberrant tumors did not differ from the other gastric cancer subtypes in the absolute T cell number, TP53 aberrant tumors harbored a decreased ratio of CD3+/FOXP3+ and CD8+/FOXP3+ T cells. The worse prognosis associated with the TP53 subtype could partly be explained by immunosuppression promoting TME. The essential biological mechanisms driving immunosuppressive TME in the TP53 subtype gastric cancer are unknown; it could be the *P53* mutation itself, including chromosomal instability. A similar finding was also seen in the non–luminal breast cancer subtype, where the low average CD8+/FOXP3+ was associated with a worse prognosis (Liu et al., 2011).

Previous studies have shown that MMR-D cancers harbor increased T lymphocytes. Most of these results were demonstrated in colorectal or endometrial cancers, although a similar association has also been proposed for gastric cancer (Eggink et al., 2017; Narayanan et al., 2019; Ratti et al., 2018; Ahtiainen et al., 2019). In contrast to previous studies, our results showed that MMR-D subtype tumors did not differ statistically in the absolute number of T lymphocytes from other molecular GC subtypes, although a trend toward high values of CD8+ T lymphocytes was detected. A possible explanation for our different results may be the relatively small number of tumors with this distinct subtype in our cohort. However, some of the

earlier published studies lacked statistical analyses or did not show increased T lymphocytes (Challoner et al., 2020; Cho et al., 2018). A study by Xing et al. showed an increased number of CD3+ lymphocytes but not CD8+ lymphocytes in MMR-D tumors compared with MMR-P tumors (Xing et al., 2018).

6.3 Formin protein in gastric cancer (III)

Formins are a group of Rho-GTPs effectors, which control cellular polarity, adhesions, and morphogenesis. Formins control many physiological or pathological processes (Kühn & Geyer, 2014). The contribution of formins to cell migration and invasion is well established in many experimental cancer models; however, their expression and role in gastric cancer (GC) remain unexplained.

In study III, the presence of two formins, FHOD1 and FMNL1, in intestinal-type GC was investigated, and their expression was correlated with clinical and molecular parameters. Previously, some studies have utilized GC cell lines and publicly available databases to analyze the role of formins in gastric cancer (Nie et al., 2020; Zhong et al., 2018). However, according to the best of our knowledge, our work is the first to utilize a significant cohort of clinical samples to study the presence of these formins in human GC specimens.

Our analysis detected the cytoplasmic expression of FHOD1 and FMNL1 along actin filaments using GC cell lines. These formins' expression patterns and localization align with previous reports of other cancer cell types (Higa et al., 2019; Peippo et al., 2017). The cytoplasmic expression was characteristic and preserved in the gastric tissue clinical samples, further confirming the integrity of the immunohistochemical staining.

Moreover, FHOD1 was strongly expressed in endothelium and plasma cells, and FMNL1 was expressed in lymphocytes and macrophages. This finding is in line with the expression profile of the two formins in an earlier study (Favaro et al., 2003) and supports the knowledge that FHOD1 and FMNL1 are likely to play a physiological role in these cell types.

In our cohort, FHOD1 expression was typically weak or absent in the non-neoplastic gastric mucosal tissue adjacent to cancer compared to the tumor tissue. This result was also confirmed in a recent study by Jiang et al. (2021). The authors investigated FHOD1 expression in a relatively small number of GC samples (N=30), and they concluded that FHOD1 expression in GC was significantly elevated compared to the expression in the adjacent non-neoplastic mucosal tissue (Jiang et al., 2021).

Previous data showed that GC molecular subtypes are known to differ in intratumoral T lymphocyte infiltration (Cho et al., 2018; Mathiak et al., 2017), and intratumoral lymphocytes are an important biological marker for tumor progression and prognosis in intestinal GC molecular subtypes (Kim et al., 2019b; Lazăr et al., 2018).

Therefore, we tested the possible correlation of formins and intratumoral lymphocytes among the different molecular subtypes GC. Indeed, we found a correlation between tumor cell FHOD1 expression and high numbers of CD8 + lymphocytes. On the other hand, we could not detect any association between cancer cell FMNL1 expression and the number of infiltrating lymphocytes. Among the GC molecular subtypes, we found reduced expression of FHOD1 in TP53 aberrant tumors. TP53 subtypes harbor lower T lymphocytes than wild-type tumors, possibly reflecting an immunosuppression environment of the TP53 mutant tumors. Alterations in intracellular signaling partially explained the low expression of FHOD1 among the TP53 mutant tumors could be partially explained by alterations in intracellular signaling. One of the commonly altered pathways in TP53-aberrant GC is the phosphatidylinositol-3-kinase (PI3K - AKT), which is crucial in controlling FHOD1 expression. While the exact mechanism controlling the TP53 and FHOD1 remains unclear, the reciprocal links await further examination.

The role of formins as a prognostic biomarker is controversial; some studies revealed a high level of FHOD1 expression was associated with poor outcomes in basal breast cancer and melanoma, while a recent study showed that the level of FHOD1 expression did not show any prognostic association in patients suffering from glioblastoma (Heuser et al., 2018, 2020). Our results showed that elevated transcript expression of FHOD1 and FMNL1 was associated with poor outcomes; this result was in line with a recent study that used bioinformatics and online databases to investigate the prognostic effect of formins in gastric cancer (Nie et al., 2020). However, our study could not recapitulate this result when we used the immunohistochemistry method in studying FHOD1 and FMNL1 protein expression in the clinical samples. The difference may be explained because FHOD1 and FMNL1 are expressed exceedingly in lymphocytes and macrophages, and the RNA analyses are typically performed from bulk tissue material, consisting of both cancer cells and stromal cells. Thus, mRNA material obtained from cells mentioned above can override the cancer cell-specific mRNA expression. At the same time, in our approach, and by using the IHC method, we only included the cancer-specific FHOD1 and FMNL1 protein expression in our analyses. Although we could not detect any direct prognostic significance for FHOD1 or FMNL1 in our study, a significant association between FMNL1 expression and tumor size and disease stage was seen. Also, FHOD1 expression was elevated in larger tumors, but our result was not statistically significant. These results indicate that FMNL1 and FHOD1 could have a role in GC tumor progression. A similar association has been detected in other cancers like basal breast cancer, melanoma, and glioblastoma multiform (Gardberg et al., 2014; Heuser et al., 2018, 2020; Nie et al., 2020).

7 Summary/Conclusions

7.1 Evaluation of the importance and applications of the results

This thesis consists of three articles, each of which has increased our understanding of gastric cancer and may have clinical implications. First, this thesis provides relevant information aiding GC stratification into biological subtypes with methods applicable to routine laboratories. The second identifies determinants associated with immune cell infiltration in GC subtypes, determining the patient´s response to immune-oncological treatments. For the third, our study on formins provides novel insight into the expression of FHOD1 and FMNL1 in GC. Both formins are linked to EMT, cancer dissemination, and progression *in vitro*, but they have not been earlier studied in the clinical context. The increasing knowledge of formins raises the possibility of targeting biomarkers in cancer treatment, including GC. Two formin targeting molecules were recently introduced; these are referred to as intramimics 1&2 (IMM-01 and-02). They are small molecule inhibitors of formin-mediated actin assembly and act as disruptors of the DID and DAD binding activation, which is responsible for the release of FH2 and activation of mDia (Lash et al., 2013). In *in vivo* experiments, IMM-01 and -02-control tumor growth in a xenograft model of colon cancer in mice (Lash et al., 2013). These molecules are non-specific but could be developed and possibly utilized as a part of GC therapy. However, further studies await whether these novel biomarkers could serve as potential drug targets in GC.

7.2 Strength and limitations

In study I analysis, we used immunohistochemistry and ngTMA methodology in molecular classifications of GC. In this method, samples of small punch diameter could be a potential source of error; in particular, the indirect detection of microsatellite status and TP53 mutations by using IHC instead of direct mutational investigations increases the risk of the error. However, immunohistochemistry is a valuable, simple, and reliable screening method in assessing MSI status in gastric cancer compared to other sophisticated methods like PCR and NGS. Recently, ESMO recommendations indicate the use of IHC as the first test of choice to test MMR proteins

in cancer; simultaneously, molecular investigation of the MMR phenotype is obligatory confirmation if IHC is uncertain (Luchini et al., 2019). On the other hand, IHC of the p53 protein testing can be used as a simplistic surrogate marker of *TP53* mutations.

Nevertheless, IHC on a small tissue sample could underestimate the protein expression level (Hwang, 2020). However, the strength of our analysis we used the four markers in detecting the protein level of the MMR status; in addition, we are likely to overcome the sample heterogeneity or underestimation of the protein level by analyzing multiple samples of the same tumor from different locations. As a control, the adjacent non-neoplastic mucosal lining was also included.

Most of the knowledge about the prognostic value of intratumoral lymphocytes and their association with tumor progression in GC originates from patients of Asian origin, with somewhat different epidemiology and genetic risk factors. Therefore, our study on European patients provides valuable complementary information. Furthermore, study II results included more detailed information about the intratumoral T lymphocytes infiltration among the intestinal-type GC molecular subtypes. In our analyses, we had the immunogenic EBV and MMR-D subtypes and other subtypes. Our novel results on the TP53 subtype could help develop new biomarkers specific for the TP53 subtype treatment.

Our study describes novel data on formin expression and cellular localization. To the best of our knowledge, our work on formins analysis and its expression patterns among the intestinal type-gastric cancer molecular subtypes is the first study utilizing clinical samples and cell lines. One of the significant obstacles in studying formins has been the incomplete knowledge of formin expression in normal and pathological tissue. The lack of well-characterized antibodies suitable for tissue immunohistochemistry increases the challenges, and this knowledge is essential to draw clinical conclusions. Thus, the strength of our work was that the formin antibodies used in study III had been validated, and their expression is characterized in human tissue in our lab (Gardberg et al., 2013, 2014; Heuser et al., 2018, 2020; Peippo, 2017).

An essential challenge in our study was that our work is a single-center study, including a relatively small sample size, which could be an obstacle when evaluating new biomarkers. Especially when multiple clinical endpoints, such as OS and RFS are included, confirmatory large-scale, multicenter studies, including large tumor sample numbers, are needed to convert these results into clinically relevant information.

7.3 Conclusions and future prospective

Gastric cancer is a heterogeneous disease, and therefore, the current uniform treatment used in all patients seems suboptimal. The novel molecular classification of gastric cancer provides the essential knowledge that improves our understanding of gastric cancer biology and will likely open new outlines for personalized medicine. Thus, the need for specific treatment of GC is in dire need of additional clinical biomarkers. Furthermore, for the standard molecular classification of GC, we need a straightforward method applicable to pathology laboratories. Thus, based on the outcomes of our research project, the conclusions are as follows:

1. The intestinal-type gastric adenocarcinoma can be classified into non-overlapping subtypes, including the EBV+, MMR-D, TP53 aberrant, and "others" subtype, each with unique clinical and biological characteristics by using a simple methodology. This methodology combines the traditional histological classifications by Laurén with immunohistochemistry and in situ hybridization analyses. This simple algorithm could provide specific information for clinical and research purposes and improve gastric cancer patients' treatment strategies.
2. T lymphocytes, mainly CD3+ and CD8+, are prognostic predictors associated with survival in intestinal-type molecular subtypes of gastric cancer. EBV+ cancers have the highest infiltrating T lymphocytes among the four molecular subtypes, suggesting that EBV+ gastric cancer patients may be suitable candidates for modern immunotherapy.
3. The study of formin protein expression in gastric cancer molecular subtypes and their relation with intramural T lymphocyte infiltration added a piece of information on the participation of FMNL1 and FHOD1 among intestinal-type molecular GC subtype tumors. However, the exact clinical significance of the upregulations of formins in GC needs further analysis.

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