The tumor immune microenvironment and clinical outcome in patients with esophageal and gastric adenocarcinoma

Svensson, Maria C

2021

Document Version:
Publisher's PDF, also known as Version of record

The tumor immune microenvironment and clinical outcome in patients with esophageal and gastric adenocarcinoma

MARIA C SVENSSON
DEPARTMENT OF CLINICAL SCIENCES, LUND | FACULTY OF MEDICINE | LUND UNIVERSITY

The tumor immune microenvironment and clinical outcome in patients with esophageal and gastric adenocarcinoma

The tumor immune microenvironment and clinical outcome in patients with esophageal and gastric adenocarcinoma

Maria C Svensson

LUND
UNIVERSITY

DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended in the Belfrage Lecture Hall, D15, 3rd floor, BMC, Lund
on December 17th at 9.00 am.

Faculty opponent
Professor Lars Andreas Akslen, University of Bergen, Norway

Organization LUND UNIVERSITY Department of Clinical Sciences Lund, Faculty of medicine, Lund University, Sweden Author: Maria C Svensson	**Document name** **Doctoral dissertation** **Date of issue** **December 17th 2021** Sponsoring organization

Title and subtitle

The tumor immune microenvironment and clinical outcome in patients with esophageal and gastric adenocarcinoma

Abstract

Tumor-infiltrating immune cells have emerged as key players in the elimination and control of tumoriginesis. Esophageal and gastric (EG) adenocarcinomas are both cancers with poor prognosis. However, the addition of chemotherepay to surgery has improved the survival rates for patients with resectable disease. The overarching aim of this thesis was to map the tumor immune microenvironment in EG adenocarcinoma and to identify immune markers for improvement of prognosticaion and response prediciton.

Expression of different immune markers were assessed by immunohistochemistry on tissue microrrays with primary tumors (PT) and paired lymph node (LN) metastases from two different patient cohorts: Cohort I encompassed 174 patients treated with surgery up-front and cohort II encompassed 148 patients treated with neaodjuvant chemotherapy (NAC) ± adjuvant chemotherapy. Paper I and II are based on cohort I, and paper III and IV are based on cohort II.

In Paper I, the infiltration of T cells and natural killer (NK) cells was investigated, with particular refererence to their prognostic impact in relation to B cells and plasma cells. The results demonstrated that high infiltration of T cells and NK cells were independent favorable prognostic factors, foremost in combination with high B cell infiltration.

In Paper II, the expression of programmed death receptor 1 (PD-1) on immune cells (IC) and programmed death receptor ligand 1 (PD-L1) on IC and tumor cells (TC) was examined in PT and paired LN metastases, along with mismatch repair (MMR) status. The results demonstrated that PD-L1 expression on IC was higer in LN metastases compared to PT, correlated with MMR deficiency (dMMR), and was an independent predictor of prolonged survival. PD-L1 expression on TC correlated with dMMR but was not prognostic. PD-1 did not correlate with MMR status, and was only prognostic in unadjusted analysis.

In Paper III and Paper IV, the effect of NAC on B cells, T cells, PD-L1 expression and different macrophage subsets was examined in paired biopsies pre-NAC and resected PT and LN metastases post-NAC. The results demonstrated that NAC appears to have the ability to alter the density as well as prognostic impact for certain immune cell subsets. No associatons were found between any immune marker in pre-treatment biopsies and histopathological regression.

Key words Esophageal cancer, gastric cancer, adenocarcinoma, tumor immune microenvrionment, prognosis, prediction, T cells, B cells, NK cells, PD-L1, PD-1, tumor-associated macrophages

Classification system and/or index terms (if any)

Supplementary bibliographical information		**Language** English
ISSN and key title 1652-8220		**ISBN**
Recipient's notes	**Number of pages 82**	Price
	Security classification	

Signature  Date 2021-11-11

The tumor immune microenvironment and clinical outcome in patients with esophageal and gastric adenocarcinoma

Maria C Svensson

LUND
UNIVERSITY

The research in this thesis was supported by the Swedish Cancer Society, the Swedish Research Council, the Mrs Berta Kamprad Foundation, the Swedish Governmental Funding of Clinical Research within the National Health Service (ALF), Skåne University Hospital Research Grants, Lund University Faculty of Medicine, The Scientific Council Region Halland, Sparbanksstiftelsen Varberg, and Regional Grants Region Halland.

Cover created by Alexander Klun

Faculty of Medicine

Department of Clinical Sciences, Lund

ISBN 978-91-8021-160-4

ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University

Lund 2021

The beautiful thing about learning is that nobody can take it away from you

BB King

Table of Contents

Thesis at a glance

Paper	Aims	Methods	Results	Conclusions
I	To investigate the clinical impact of T cells and NK cells in relation to B cells and plasma cells in CRT-naïve EG adenocarcinoma.	Retrospective cohort Tissue microarrays Immunohistochemistry Endpoints: OS, TTR	High infiltration of T cells and NK cells were favorable prognostic factors, foremost in combination with high B cell infiltration.	These findings support that the antitumoral effects of T cells may be largely dependent on a functional interplay with B cells.
II	To examine the expression of PD-L1 and PD-1 in PT and paired LN metastases in CRT-naïve EG adenocarcinoma, along with MMR status and prognosis.	Retrospective cohort TCGA Tissue microarrays Immunohistochemistry In situ hybridization Endpoints: OS, TTR	PD-L1 expression on IC was higher in LN metastases compared to PT, correlated with MMR deficiency and was an independent factor of prolonged survival. PD-L1 expression on TC was not prognostic.	The prognostic value of PD-L1 expression is only attributed to its expression on IC. The heterogeneity of PD-L1 expression is also highlighted.
III	To assess the effect of NAC on the density and prognostic impact of B cells, T cells and PD-L1 expression on paired biopsies pre-NAC and resected PT and LN metastases post-NAC in EG adenocarcinoma.	Retrospective cohort Tissue microarrays Immunohistochemistry Endpoints: OS, TTR Histopathological response	$CD8^+$ T cells increased and $FoxP3^+$ T cells decreased after NAC. High $FoxP3^+$ density and high $PD\text{-}L1^+$ IC expression were beneficial prognostic factors pre-NAC, whereas high $CD8^+$ density was an unbeneficial prognostic factor. High $FoxP3^+$ density and high $PD\text{-}L1^+$ TC exression were adverse prognostic factors post-NAC.	NAC may have the ability to alter the density, prognostic impact and possibly even the functional competence of certain IC subsets.
IV	To examine the effect of NAC on the density and prognostic significance of TAM subsets in paired biopsies pre-NAC and PT and LN metastases post-NAC in EG adenocarcinoma.	Retrospective cohort Tissue mircoarrays Immunohistochemistry Endpoints: OS, TTR Histopathological response	$CD68^+/CD163^+$ TAM density increased and $MARCO^+$ TAM density decreased after NAC. High $CD68^+/CD163^+$ TN infiltration was an unfavorable prognostic factor pre-NAC. High total and high TN infiltration of $CD68^+/CD163^-$ TAM were adverse prognostic factors post-NAC.	NAC may have the ability to alter the density of certain TAM subsets along with their functional competence and, thus, their prognostic value.

Abbreviations: CRT – chemoradiotherapy; EG – esophageal and gastric; OS – overall survival; TTR – time to recurrence; PT – primary tumors; LN – lymph node; MMR – mismatch repair; TCGA – The Cancer Genome Atlas; IC – immune cells; TC – tumor cells; NAC – neoadjuvant chemotherapy; TAM – tumor-associated macrophages; TN – tumor nest

Papers included in the thesis

The following papers form the basis of the thesis, and are referred to by their Roman numerals in the text.

I. **Svensson MC**, Warfvinge CF, Fristedt R, Borg D, Hedner C, Eberhard J, Micke P, Nodin B, Leandersson K, Jirström K. The integrative clinical impact of tumor-infiltrating T lymphocytes and NK cells in relation to B lymphocytes and plasma cell density in esophageal and gastric adenocarcinoma. *Oncotarget* 2017;8:72108-72126

II. **Svensson MC**, Borg D, Zhang C, Hedner C, Nodin B, Uhlén M, Mardinoglu A, Leandersson K, Jirström K. Expression of PD-L1 and PD-1 in chemoradiotherapy-naïve esophageal and gastric adenocarcinoma: relationship with mismatch repair status and survival. *Frontiers in Oncology* 2019;9:136

III. **Svensson MC**, Lindén A, Nygaard J, Borg D, Hedner C, Nodin B, Leandersson K, Jirström K. T cells, B cells and PD-L1 expression in esophageal and gastric adenocarcinoma before and after neoadjuvant chemotherapy: relationship with histopathological response and survival. *Oncoimmunology* 2021;10e: 1921443

IV. **Svensson MC**, Svensson M, Nodin B, Borg D, Hedner C, Hjalmarsson C, Leandersson K, Jirström K. High infiltration of $CD63^{+}/CD168^{-}$ macrophages is an adverse prognostic factor after neoadjuvant chemotherapy in esopahgeal and gastric adenocarcinoma. *Submitted*

Papers not included in the thesis

- Berntsson J, Svensson MC, Leandersson K, Nodin B, Micke P, Larsson AH, Eberhard J, Jirström K. The clinical impact of tumour-infiltrating lymphocytes in colorectal cancer differs by anatomical subsite: a cohort study. *International Journal of Cancer* 2017;141:1654-1665

- Jeremiasen M, Borg D, Hedner C, Svensson M, Nodin B, Leandersson K, Johansson J, Jirström K. Tumour-associated $CD68^{+}$, $CD163^{+}$ and $MARCO^{+}$ macrophages as prognostic biomarkers in treatment-naive oesophageal and gastric adenocarcinoma. *Frontiers in Oncology* 2020;10:534761

Abbreviations

APC; Antigen presenting cells

BCR; B cell receptor

Bregs; B regulatory cells

CPS; Combined positive score

CRT; Chemoradiotherapy

CTL; Cytotoxic T cells

CTLA-4; Cytotoxic T-lymphocyte-associated protein 4

DIA; Digital image analysis

dMMR; Deficient mismatch repair

EBV; Epstein-Barr virus

ECF; Epirubicin, cisplatin and fluorouracil

EG adenocarcinoma; Esophageal or gastric adenocarcinoma

EG cancer; Esophageal and gastric cancer

EG junction; Esophago-gastric junction

EMA; European Medicines Agency

EOX; Epirubicin, oxaliplatin and capecitabine

EPV; Events per predictor variable

FDA; Food and Drug Administration

FFPE; Formalin fixed paraffin embedded

FLOT; Fluorouracil, leucovorin, oxaliplatin and docetaxel

FoxP3; Forkhead box P3

HER2; Human epidermal growth factor receptor 2

ICI; Immune checkpoint inhibitor

IFNγ; Interferon-γ

IGKC; Immunoglobulin kappa C

IHC; Immunohistochemistry

IL; Interleukin

MARCO; Macrophage receptor with collagenous structure

MHC; Major histocompatibility complex

MSI-H; MSI-High

MSI; Microsatellite instability

MSS; Microsatellite stable

NAC; Neoadjuvant chemotherapy

NK; Natural killer

NKG2D; NK receptor member D of the lectin like receptor family

NKT; Natural killer T

OS; Overall survival

PD-1; Programmed death receptor 1

PD-1_{IC}; PD-1 immune cell

PD-L1; Programmed death receptor ligand 1

PD-$L1_{IC}$; PD-L1 immune cell

PD-$L1_{TC}$; PD-L1 tumor cell

PD-L2; Programmed death receptor ligand 2

pMMR: Proficient mismatch repair

SCC; Squamous cell carcinoma

TAM; Tumor-associated macrophages

TCGA; The Cancer Genome Atlas

TCR; T cell receptors

TLS; Tertiary lymphoid structures

TMA; Tissue microarray

TME; Tumor microenvironment

TN; Tumor nests

TNFα; Tumor necrosis factor α

Tregs; T regulatory T cells

TRG; Tumor regression grading

TRM; Tissue-resident macrophages

TTR; Time to recurrence

Introduction

Cancer has afflicted us since ancient times and the oldest description descends from Egyptian manuscripts written between 1500-1600 B.C. (1). Since then, the prevalence of cancer has increased remarkably. This is due to numerous factors including growing populations, higher age, increasing risky health behavior and presence of carcinogens in the environment (1). Moreover, some cancers are linked to infections and others to heredity. We know today that there are certain rules that dictate the transformation of normal cells into malignant tumors, so called hallmarks of cancer, as summarized in two pioneering papers by Hanahan *et al.* in 2000 and 2011 (2, 3). The first six hallmarks of cancer comprise the following characteristics; self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, enabling replicative immortality, inducing angiogenesis and activating tissue invasion and metastasis (2). All these capabilities are acquired during tumorigenesis and are thought to be shared in all different types of tumors (2). However, with a growing understanding of cancer, including the insight that not solely the cancer cell itself, but also its surrounding microenvironment, is of paramount importance for tumorigenesis, two emerging hallmarks were proposed; deregulation of cellular metabolism and evasion of immunological destruction, as well as two enabling characteristics; genomic instability and tumor-promoting inflammation (3). The biology of cancer is highly complex and significant advances in research are still needed to improve prognostication as well as treatment prediction for this versatile disease.

This thesis investigates the prognostic and potential predictive role of the tumor immune microenvironment in esophageal and gastric adenocarcinoma.

Background

The immune system in cancer

Cancer is an insidious disease, and the presence of inflammatory immune cells in the tumor microenvironment (TME) has raised fundamental issues in cancer research. The elimination and control of malignant cells by the immune system is referred to as immune surveillance. However, tumor cells can develop several mechanisms to avoid recognition and destruction, which is referred to as immune evasion. The interaction between the immune system and cancer is highlighted as one of the hallmarks of cancer, defined by Hanahan *et al*., where immune evasion and tumor promoting inflammation are key features of all cancer types (2, 3). These hallmarks are illustrated in Figure 1.

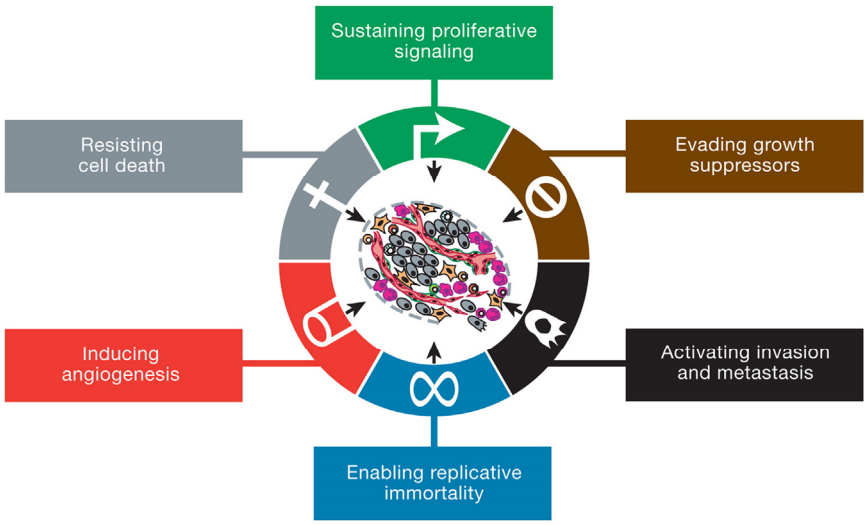


Figure 1.
Illustration of the six hallmark capabilities. Reprinted from Hanahan et al. (3), copyright with permission from Elsevier.

The TME is the microenvironment of the tumor and consists of extracellular matrix, fibroblasts, endothelial cells, signaling molecules and immune cells. The immune system, in turn, consists of two distinct compartments; the innate and the adaptive

immune response. The innate immune response is a rapid, semi-specific, first line defense against foreign pathogens while the adaptive immunity is characterized by a high antigen-specificity, diversity and immunological memory (4). Clinical and experimental studies have demonstrated both innate and adaptive immune cells to be of great importance in immunoediting, although paradoxical and context depending (5).

The innate and adaptive immune system

Apart from immune cells, the innate immune system also involves various barriers; epithelial (e.g. skin, mucosal membranes), as well as chemical (e.g. gastric acid, saliva). The cellular components comprise mast cells, neutrophils, basophils, eosinophils, dendritic cells, macrophages, natural killer (NK) cells, innate lymphoid cells and semi-innate lymphoid cells such as natural killer T (NKT) cells. These cells have a wide range of skills and contribute to tumor suppression by direct recognition of deviating molecular patterns on tumor cells. This in turn optimally leads to elimination of tumor cells and activation of the adaptive immune response.

The adaptive immune system can be divided into the cell-mediated and the humoral mediated immunity. The former consists of T cells, and the latter of antibodies produced by the B cells (plasma cells). T cells and B cells are lymphocytes of various lineages, subtypes, functions and phenotypes. The adaptive immunity is multifaceted, however the main functions in tumor immunology can be described as; acquiring antigen specific receptors, recognizing tumor antigens (i.e. aberrant antigens), specific killing of the antigen expressing tumor cells, producing antibodies with high specificity for these antigens, and forming immunological memory.

Tumor-associated macrophages

In 1908, Ilja Metjnikov was one of two Nobel Prize laureates in physiology or medicine for the discovery of cellular immunology and phagocytosis (6). Metjnikov was the first to describe macrophages and their ability to engulf and eliminate cellular components from living and dead host cells (7).

Macrophages that originate from the yolk sac and fetal liver are referred to as tissue-resident macrophages (TRM). These macrophages are self-maintaining and capable of local proliferation without help from monocyte-derived macrophages. They can be long-lived in some tissues such as brain, liver and lung, while in other tissues they are replaced by monocyte–derived cells. The main task of TRM is to facilitate tissue homeostasis by e.g. clearing apoptotic cells, responding to pathogens or toxins, and stimulating activation, proliferation and differentiation of other immune cells (8-10).

Bone marrow derived macrophages arise from the mononuclear myeloid lineage. They circulate the blood as monocytes and differentiate into macrophages in the tissues. Their primary role is to phagocyte cells and act as antigen presenting cells (APC), but they are also involved in a wide range of processes including inflammation, immunosuppression, angiogenesis and tissue repair (11, 12).

Macrophages are considered frontier soldiers of the innate immunity and are highly important at sites of inflammation where they secrete numerous cytokines such as tumor necrosis factor α (TNFα), interleukin (IL) 1, IL 6, IL 12 and nitric oxide. The release of these inflammatory mediators activates the defense against microbes and aids in killing them. While the effect of this first line defense mechanism is initially beneficial, it also contributes to major tissue damage, and in order not to become harmful, macrophages either undergo apoptosis or switch to an anti-inflammatory phenotype (12). This behavioral variation reflects the dual role of macrophages that is also evident in the TME.

Tumor-associated macrophages (TAM) are the most abundant immune cell in the TME, and their functional state depends on the type of activation, resulting in two extreme subtypes referred to as M1 (classically activated) and M2 (alternatively activated) (13, 14). The M1 phenotype is associated with antigen presentation, production of pro-inflammatory cytokines (e.g. interferon-γ (IFNγ), IL 6, IL 12 and TNFα, generation of reactive oxygen species and microbicidal and tumoricidal activity. The M2 phenotype is associated with production of anti-inflammatory cytokines (e.g. IL 10), remodeling of tissues and upregulation of scavenger receptors. The tumor-suppressive role of TAM involves recruitment and activation of cytolytic cells, such as NK cells and cytotoxic T cells while the tumor-promoting role of TAM includes inhibition of cytotoxic T cells, promoting regulatory T cells and inducing wound healing mechanisms leading to invasion and angiogenesis (13-15). It has been suggested that the tumor milieu itself initiates the differentiation of monocytes towards either tumor-suppressing (M1) or tumor-promoting (M2) subtypes (16).

A scavenger receptor expressed by some TAM subsets, is the macrophage receptor with collagenous structure (MARCO). When this receptor is activated, phagocytosis is mediated (17). The prognostic impact of $MARCO^+$ TAM is highly uninvestigated and with ambiguous results. In hepatocellular carcinoma an adverse association between decreased intratumoral expression and survival was demonstrated (18). Contrary, in intestinal-type periampullary carcinoma, high density was an adverse prognostic factor (19). To the best of our knowledge, only one former study has examined the prognostic impact in esophageal or gastric (EG) adenocarcinoma, in a cohort of chemoradiotherapy-naïve patients, whereby no association with survival was identified (20).

Considering the fact that macrophages exist on a broad spectrum and demonstrate a functional and phenotypic plasticity, the view of TAM as either of the extreme

subtypes (M1 or M2) is an oversimplification (15).This needs to be kept in mind when investigating their prognostic role in cancer. However, total TAM infiltration is in general associated with poor survival in several tumor entities, including esophageal and gastric (EG) cancer (21-24), but contrasting findings have been reported in colon cancer (25).

NK/NKT cells

NK cells are lymphocytes that originate from the same lymphoid progenitor as T cells, B cells and NKT cells. They have both cytokine releasing and cytotoxic effector functions, however, unlike cytotoxic T cells, they do not have clonally distributed specificity for antigens or immunological memory (26). The tumoricidal effect of NK cells can be due to upregulation of ligands for NK cell receptors and/or loss of major histocompatibility complex (MHC) I on tumor cells (27).

The activation of NK cells is determined following a combination of stimulatory and inhibitory signals. Activating NK receptors are e.g. NK receptor member D of the lectin like receptor family (NKG2D) and the cytotoxicity receptor NKp46 (28). NKG2D ligands are upregulated on stressed cells, such as infected, damaged or malignant cells, while poorly expressed on normal cells. Inhibiting NK receptors are activated when encountering MHC I molecules. The expression of MHC I is lower in tumor cells than in normal cells and the incapacity to present self MHC I molecules is referred to as “missing self” and results in NK cell activation (27, 29, 30).

The effector functions of NK cells can be summarized by the release of cytoplasmic granules containing cell lysing proteins, the expression of tumor necrosis factor inducing tumor-cell apoptosis, Fas:FasL induced cell death, and the production of cytokines such as IFN-γ which invigorate adaptive immunity (31).

High density of tumor infiltrating NK cells has been shown to be associated with improved prognosis in many types of cancer, including EG cancer. Contrasting results have however been reported, depending on which NK marker has been investigated, as well as the tumor type (32-34). Furthermore, it has been suggested that the tumor milieu itself can drive the NK cell to an anergic state, hence enabling the tumor to evade elimination (35).

NKT cells are a subset of lymphocytes that share features of both NK cells and T cells. This is illustrated by the fact that they express T cell receptors (TCR), T cell markers (CD3, CD8) as well as NK cell markers (CD56, NKp46, NKG2D). Unlike T cells, that recognize MHC proteins, or NK cells, that recognize missing self-MHC, NKT cells recognize lipid antigens presented by CD1d molecules. Upon activation, NKT cells can eliminate tumor cells by a direct cytotoxic cell-mediated lysis, but their foremost function is to mediate cytokine release and an immunomodulating effect, which results in an enhanced B and T cell response (36, 37). The prognostic

impact of tumor infiltrating NKT cells in EG cancer is largely unexplored, but low infiltration has been associated with poor survival in gastric cancer (38).

T cells

T cells are characterized by expression of TCR which bind to MHC class I and II molecules. MHC class I molecules are displayed by all nucleated cells while MHC II molecules are displayed foremost by APC. T cells can be divided into three different groups depending on their characteristics; T helper cells ($CD3^+CD4^+$), cytotoxic T cells ($CD3^+CD8^+$) and T regulatory T cells ($CD3^+FoxP3^+CD4^+CD25^+$).

T cells originate from bone marrow lymphocyte progenitors that mature and become selected in the thymus. Thereafter they migrate to secondary lymphoid organs in which they are exposed to antigens presented by APC, and become activated.

Three main signals are needed to activate $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells. Signal one is recognition and binding of the T cell receptor to an antigen held by a MHC molecule on the APC. This initial phase also includes binding of T cell receptor molecules directly to MHC molecules, thus stabilizing the connection (39). Step two consists of secondary signals, which for CD4 helper T cells in particular, but also for CD8 cytotoxic T cells, include binding of CD28 molecules to either B7.1 (CD80) or B7.2 (CD86) on the APC, leading to a massive clonal T cell proliferation. In order to regulate this proliferation, regulatory co-receptors like cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (CD152) are present, which in turn competes with CD28 for B7 binding and thus promotes a regulation of the immune response. The third signal in T cell activation is carried out by cytokines and is necessary for the regulation of T cell differentiation and effector capacities (40, 41).

The main function of T helper cells is to enhance and direct the function of other immune cells such as cytotoxic T cells (CTL) and B cells. This indirect promotion is provided through the secretion of cytokines like IFNγ and IL-2, which amplifies the activation and expansion of $CD8^+$ T cells. However IFNγ is also provided by CTLs, NK cells and macrophages (42). Another major way $CD4^+$ T cells support $CD8^+$ T cells is by enhancing the ability of dendritic cells to present antigens and co-stimulatory signals. These features are necessary for the interaction between dendritic cells and $CD8^+$ T cells (43).

Cytotoxic $CD8^+$ T cells can be described as guided missiles of the anticancer immune response, as they eliminate cells by specific recognition of tumor antigens leading to the effector phase with secretion of cell lytic granzymes and perforin. CTL also have the capability to secrete cytokines such as IFNγ and TNFα (43). As previously described, $CD4^+$ T cells are highly important for the maintenance of the $CD8^+$ T cell response, but they are also important for avoidance of exhaustion (44). Exhaustion is induced following a consistent exposure of tumor antigens which

leads to a persistent expression of inhibiting immune-checkpoint molecules such as programmed death receptor 1 (PD-1). This in turn impairs the effector and proliferative functions of immune cells, leading to immunosuppression and a failure in tumor surveillance (44, 45).

Another noteworthy phenomenon in the tumor microenvironment is T cell anergy. This may develop when the T cell receives a TCR:antigen/MHC signal, but no sufficient co-stimulatory signals. This results in T cell tolerance for specific antigens. This mechanism is associated with a reduced IL-2 production (46).

T regulatory T cells (Tregs) are a very immuno-suppressive subset of CD4 helper cells. The chief feature of Tregs is expression of the transcription factor Forkhead box P3 (FoxP3), which is critical for their development and effector functions. $FoxP3^+$ Tregs can exert suppression by direct cell contact with APC or effector T cells, as well as by indirect contact by secreting immune modulating cytokines or by excessive consumption of IL-2 (47).

While tumor-infiltrating T cells in general, and cytotoxic $CD8^+$ T cells in particular, have been associated with improved prognosis in EG cancer, the role of Tregs is more ambiguous, but the majority of studies demonstrate an association with poor prognosis (34, 48-51).

Immune checkpoints

To ensure a valid regulation of the immune response, inhibitory receptors are expressed on the surface of immune cells (44). These receptors are jointly referred to as immune checkpoints and include e.g. PD-1 and CTLA-4. PD-1 is expressed on T cells, B cells, NK cells, NKT cells, activated monocytes and dendritic cells, but of note it is not expressed on inactive T cells. CTLA-4 is exclusively expressed on T cells (44, 52).

PD-1 is a type I transmembrane protein that binds to the ligands programmed death receptor ligand 1 (PD-L1) and programmed death receptor ligand 2 (PD-L2), both overexpressed on tumor cells and APCs in the tumor microenvironment, and by doing so, strongly prevents TCR signal transduction and CD28 co-stimulation (39). The engagement of PD-1 on $CD8^+$ T cells also leads to a switch from glycolysis to fatty acid beta-oxidation, inducing mitochondrial damage and cell death (53). Immune checkpoint blockade with e.g. antibodies, counteracts the PD-1 signal transduction, thus enhancing T cell functions (39).

PD-L1, a transmembrane protein belonging to the B7 family, is expressed on tumor cells as well as B cells, T cells, macrophages, and dendritic cells. An important stimulus for PD-L1 expression is IFNγ (54, 55). PD-L1 interacts with the receptor PD-1 as described previously.

The prognostic value of PD-L1 and PD-1 expression in EG cancer is diverse, however the latter is less investigated (56-61).

B cells

B cells are lymphocytes that develop in the bone marrow and are crucial for the adaptive immune response and humoral antibody response (62). Activation of B cells is caused by the interaction between the B cell receptor (BCR) and antigens in secondary lymphoid organs, followed by B cell antigen uptake, degradation of the captured antigen internally, and then presentation of antigen fragments on MHC class II molecules. This enables an interaction with the antigen specific $CD4^+$ TCR of T helper cells, which is a crucial step in the B cell activation process (linked recognition) (63, 64). Following this engagement, the interaction also gives rise to an immunological synapse, sanctioning a bidirectional crosstalk between the B and T cells (63). Pending this primary response, a subset of rapidly proliferating antigen specific B cells will form germinal centers (GC) in secondary lymphoid organs, wherein B cells interact with follicular helper T cells and complete the differentiation into either antibody producing plasma cells or memory cells (63, 65). CD20 is considered the main marker of B cells throughout all stages of differentiation, however, when differentiated into plasma cells, this marker is downregulated (66). Hence, plasma cells can be assessed separately and are primarily identified by the marker immunoglobulin kappa C (IGKC.) The spatial distribution of T cells and B cells in the TME has also attracted a large amount of interest recently. The formation of so called tertiary lymphoid structures (TLS) are immunohistochemically identified by colocalization of $CD3^+$ (pan-T cell marker) and $CD20^+$ (B cell marker) cells and several studies have established B cells and TLS to be highly important for the response to immune checkpoint inhibition as well as improved patient outcome, also in gastric cancer (65, 67-69).

B cells are in general considered positive immune regulators due to their ability to produce antibodies. However, other additional functions have been discovered such as regulation and cytokine production. B cells producing IL-10 are referred to as B regulatory cells (Bregs) and have an immunosuppressive capacity (70). Bregs have an immature phenotype and lack specific surface immune markers, thus they are classified based upon their cytokine production (71). Given that B cells, just like T cells, constitute different subtypes with diverse effector functions, however mostly unaccounted for, it is not surprising that the prognostic impact of B cells in the TME can be somewhat ambiguous. In EG adenocarcinoma, however, several studies have shown a correlation of B cells with favourable prognosis (34, 67, 72).

Esophageal and gastric adenocarcinoma

Incidence and epidemiology

EG cancers are together responsible for a considerable amount of cancer cases and deaths worldwide. In 2020, over 1,7 million cases were reported, together ranking them as fourth for incidence and second for cancer related mortality globally. EG cancer incidence rates are approximately twice as high in men compared to women, and the distribution of these cancers vary greatly (73).

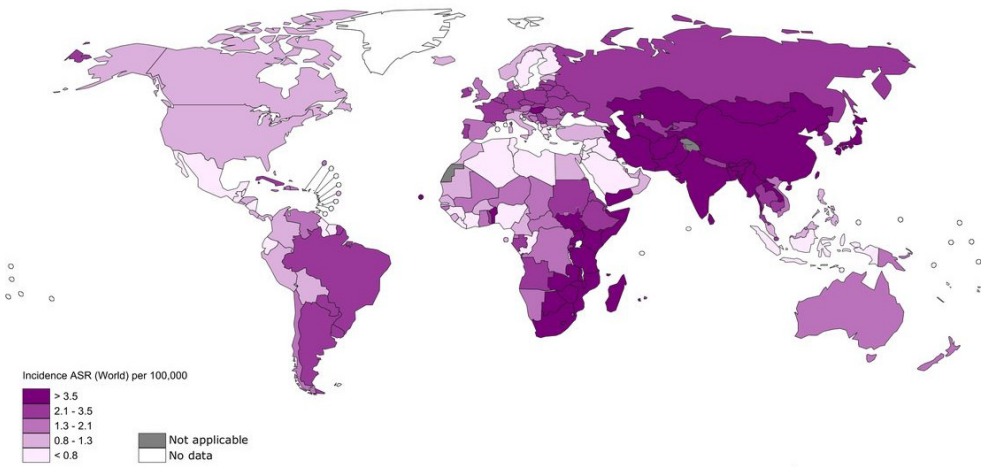
The incidence of esophageal adenocarcinoma has increased swiftly in the westernized world over the last 30 years, surpassing the previously more common subtype squamous cell carcinoma (SCC) (74, 75). Globally however, 84% of the approximately 572000 new cases of esophageal cancer in 2018 were SCCs and 15% were adenocarcinomas. Eastern Asia and Eastern Africa have the highest incidence rates of SCC while adenocarcinoma dominates in high-income countries in Northern Europe, North America and Oceania (76).

In 2018, approximately 1 million new cases of gastric cancer were recorded globally, with 70% occurring in Asia (76). Gastric cancer can anatomically be divided into cardia gastric cancer and non-cardia gastric cancer. Non-cardia gastric cancer exceeds cardia gastric cancer globally, comprising 82% of all cases, with the highest rates in Asia, followed by areas such as South and Central America. For cardia gastric cancer, the highest rates are noted in Eastern Asia as well as in parts of Oceania and Western Asia (76).

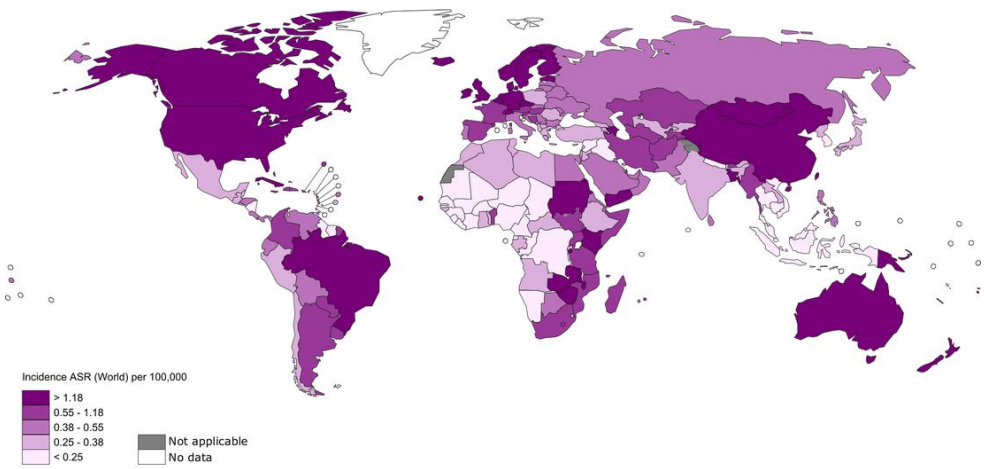
The incidence and mortality rates of non-cardia gastric cancer has declined worldwide over the past one-half century in most populations (77). The falling incidence is mostly due to the reduction of *Helicobacter pylori (H. pylori)* prevalence. It is however notable that absence of *H. pylori* in the stomach has been suggested to promote development of esophageal adenocarcinoma by increasing the concentration of e.g. acids in gastric refluxes, thus damaging the esophageal mucosa (76).

Of note, in the aforementioned epidemiological studies, cardia cancer is designated as gastric cancer, whereas in clinical practice and in recent TNM classifications at least cardia cancer type I and II (i.e. esophago-gastric junction Siewert type I-II tumors) are considered to be esophageal cancers. Thus, the incidence and prevalence rates of these cancer entities are somewhat challenging to map out, given their heterogeneous classification. Figure 2 demonstrates the global incidence of esophageal SCC, esophageal adenocarcinoma, cardia gastric cancer and non-cardia gastric cancer, respectively.

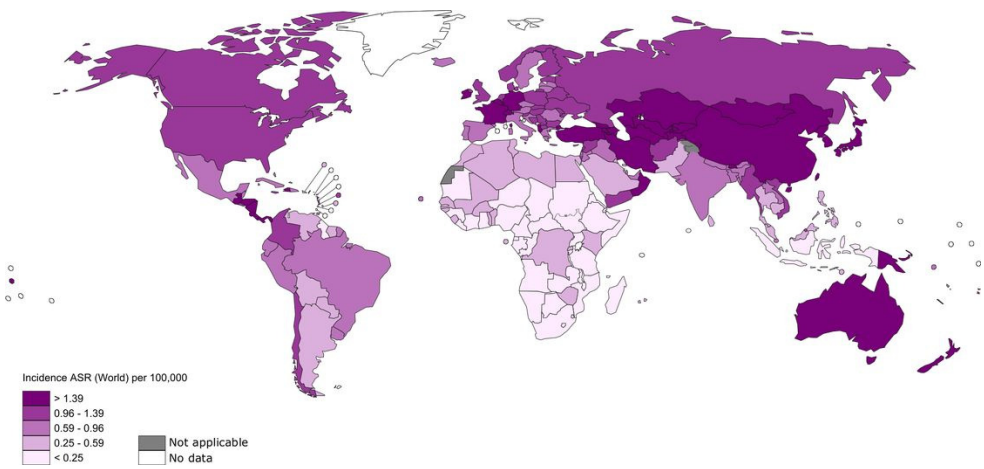
A



B



C



D

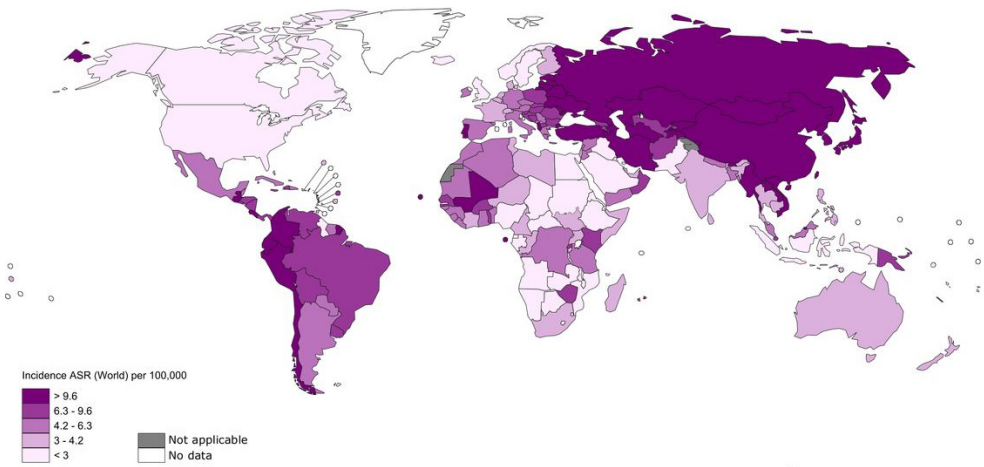


Figure 2.
Age-standardized incidence rate (ASR, World) per 100,000, both sexes combined, in 2018 for (A) esophageal SCC (B) esophageal adenocarcinoma, (C) cardia gastric cancer, and (D) non-cardia gastric cancer. Reproduced from Arnold et al. (76) with permission from BMJ Publishing Group Ltd.

Etiology

H. pylori is a gram-negative bacterium that colonizes the stomach, induces chronic inflammation, and was first isolated by Warren and Marshall in 1983 (78). The chronic inflammation is persistent unless treated and constitutes a major risk factor for intestinal type gastric cancer due to its overall prevalence, which is a little over 30% in developed countries and approximately 50% in less developed countries. It is estimated that approximately 89% of non-cardia gastric cancer cases worldwide is attributable to *H. pylori* infection (79). Environmental risk factors associated with gastric cancer is low intake of fruits and vegetables, high intake of salt, pickled food, smoking and heavy alcohol consumption (80, 81). The Epstein-Barr virus (EBV) is another known risk pathogen associated with gastric cancer, although much less common than *H. pylori,* with an estimated prevalence of 8.7% of all gastric cancer cases (82). Hereditary syndromes such as familial intestinal gastric cancer, hereditary diffuse gastric cancer and gastric adenocarcinoma and proximal polyposis of the stomach encompass approximately 1-3% of all gastric cancers. Moreover, even though most cases are sporadic, family clustering is observed in about 10% of cases (83).

A strong risk factor for developing esophageal adenocarcinoma is gastroesophageal reflux (84), as is increasing body mass index (85), but, contrary to SCC, alcohol intake is not (86). Furthermore, *H. pylori*, vegetables and fruits are suggested to be preventive factors (87, 88). Lower socio-economic status has been identified as a risk factor for both SCC and adenocarcinoma (89). Tobacco smoking is, in addition to alcohol consumption, the major risk factor for esophageal SCC. Moreover red, salted and processed meat has also been addressed as a possible risk factor (90, 91).

Anatomy

Anatomically, the esophagus stretches over 25 centimeters and can be divided into three different parts. The cervical (upper) part spans from below the hypopharynx to the thoracic inlet. The thoracic (middle) part spans from the thoracic inlet to the hiatus and the abdominal (lower) part spans from the hiatus to the esophago-gastric (EG) junction. The EG junction, in turn, stretches from 5 cm proximally to 5 cm distally of the anatomical cardia, which is also referred to as the true cardia. According to the German surgeon Siewert, tumors of the EG junction are further subdivided into three categories; distal esophageal cancer (Siewert type I), true cardia cancer (Siewert type II) and subcardial gastric carcinoma (Siewert type III) (92). The Z line is the histological zone in the EG junction where squamous cell epithelium transitions to gastric mucosa. In this thesis, only adenocarcinomas have been investigated. The stomach is anatomically divided into three parts; the upper

part is called the fundus, the middle part the corpus and the distal part the pylorus. The anatomy of the esophagus and stomach is illustrated in Figure 3.

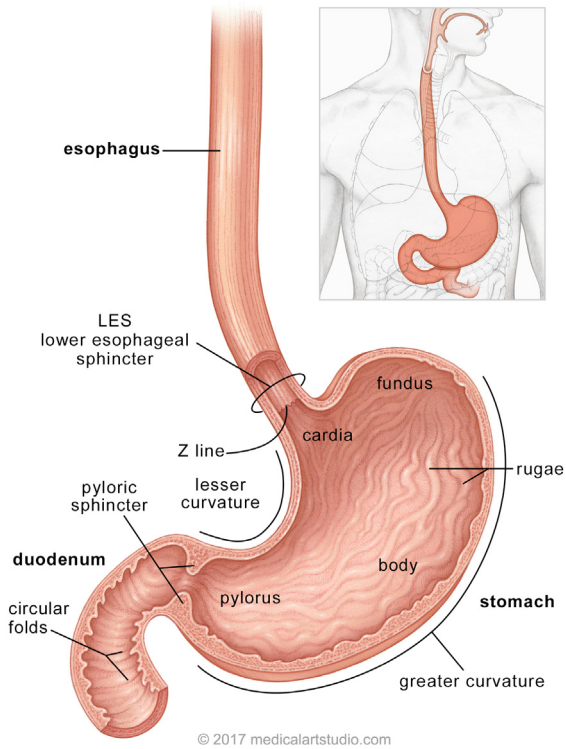


Figure 3.
Anatomy of the esophagus and stomach. Reprinted with permission from © Medicalartlibrary.com.

Classification and pathogenesis

Primary esophageal cancer constitutes over 95% of all esophageal malignancies and rare differential diagnoses are sarcoma, lymphoma and metastases (93). Esophageal cancer is commonly classified as SCC or adenocarcinoma. SCC develops following increasing grade of dysplasia and can occur at any level in the esophagus but most commonly in the middle third, followed by the lower third and the upper third (93, 94).

Adenocarcinomas are primarily located in the distal part of the esophagus including the EG junction (93). The histological classification of esophageal adenocarcinoma is sometimes further refined by applying the Laurén classification system, explained in more detail below (95).

Approximately 95% of all gastric tumors are adenocarcinomas. The vast majority of tumors are related to infectious agents while a minority is related to hereditary causes such as Lynch syndrome. Rare differential diagnoses to gastric adenocarcinoma are e.g. lymphoma, gastrointestinal stromal tumors (GIST) and neuroendocrine tumors (96).

Dating back to 1965, gastric adenocarcinoma is histologically subdivided into intestinal or diffuse type according to the Laurén classification (97). The pathogenesis differs between the two subtypes in that intestinal-type tumors arise from gastric atrophy and intestinal metaplasia in combination with environmental and dietary factors, while the diffuse-type tumors have no evident precursor lesions (98).

The intestinal type is characterized by cohesive cells which form a gland like structure. It is associated with chronic inflammation due to *H. pylori* infection, distal tumor location, older age and male sex. The diffuse type has no association with chronic inflammation, but instead a relationship to dysfunctional cell adhesion leading to populations of non-cohesive scattered tumor cells. It is associated with female sex, younger age, proximal tumor location and poor prognosis. Intestinal type is decreasing in incidence while diffuse type is increasing. The increasing incidence of the former could be a result of shifting proportions of the two subgroups, however a study from the United States investigated the trend by analyzing histopathologic data on gastric adenocarcinomas from 1973 through 2000, and concluded the increase to be true, and mainly due to the rise of signet cell ring type gastric carcinoma, included in the diffuse subgroup (99-102).

It is well known that *H. pylori* is a risk factor for gastric cancer, however a subset of gastric cancers is related to another infectious pathogen; the EBV virus. EBV-positive tumors often occur in the proximal stomach, form ulcers, display dense lymphocyte infiltration and have also been demonstrated to have a better prognosis (103). Due to recent advances in the genetic characterization of EG cancer, a new molecular classification has been developed, wherein EBV-positive tumors constitute one of the subgroups (104, 105).

The new molecular classification, which includes both esophageal and gastric cancer, was developed by The Cancer Genome Atlas (TCGA) and is an important addition to histopathology. Five different subtypes have been identified based on their specific genetic alterations using different molecular platforms; esophageal SCC (ESCC), chromosomal instability (CIN), genomically stable (GS), microsatellite instability (MSI) and EBV (104, 105), as demonstrated by Figure 4.

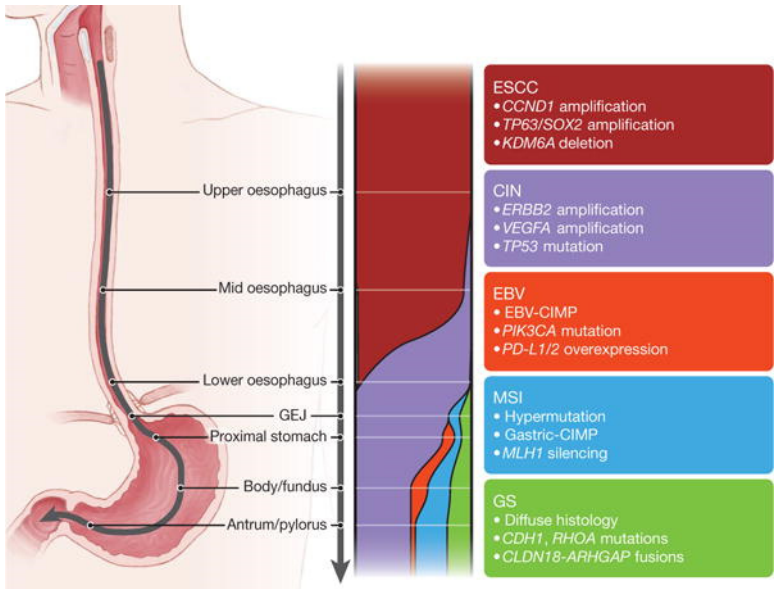


Figure 4.
TCGA molecular subtypes and key features of esophageal and gastric cancer. Reprinted from Nature (105) with permission from Springer under the terms of https://creativecommons.org/licenses/by/4.0/.

Clinical presentation and diagnosis

Symptoms of esophageal and gastric tumors are in general vague and also common in benign conditions (106). Nevertheless, the most common early symptom of esophageal cancer is dysphagia, and other less common symptoms are dyspepsia, reflux, vomiting, weight loss, dyspnea, nausea, bleeding, anemia and pain (106-108). Regarding gastric cancer, early symptoms are unusual, however any of the above described symptoms related to esophageal cancer may be present at some time point (109, 110).

Diagnosing and staging of esophageal and gastric tumors includes upper gastrointestinal endoscopy with biopsies, computed tomography (CT) of the chest, abdomen and pelvis, and in some cases also ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography (PET). In cases where peritoneal carcinomatosis is suspected, it can be of value to perform a staging laparoscopy (111). Endoscopic ultrasound may also contribute to the staging procedure by assessing the local infiltration of primary tumors and local nodal status (108).

All patients should be discussed at a multidisciplinary team meeting to ensure accurate staging and treatment recommendations.

Staging

Esophageal and gastric tumors are staged according to the UICC/AJCC TNM classification. T – category refers to the invasive depth of the primary tumor, N – category refers to the involvement of lymph node metastasis and M refers to the presence or absence of distant metastasis (Figure 5). In this thesis, clinical and histopathological classification of tumor stage was done according to the 7th edition of the UICC/AJCC TNM classification, in which EG junction Siewert type I-III tumors are classified as esophageal tumors (112), Figure 6. However, as EG junction Siewert type III tumors are managed as gastric tumors in the clinic, and according to the new 8th edition of UICC/AJCC TNM classification (113) (Table 1), this definition was used for the subgroup analyses according to tumor location in paper I-IV.

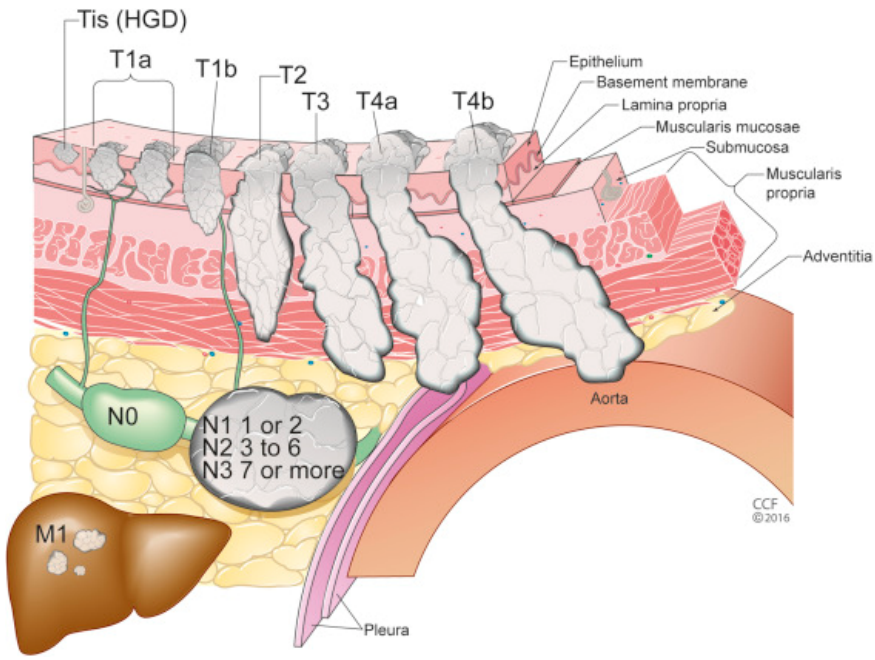


Figure 5.
Illustration of the T, N and M categories in esophageal cancer. In gastric cancer the categories are similar, however the stomach is surrounded by serosa instead of adventitia. Reprinted from Rice *et al.* (114) with permission from Elsevier.

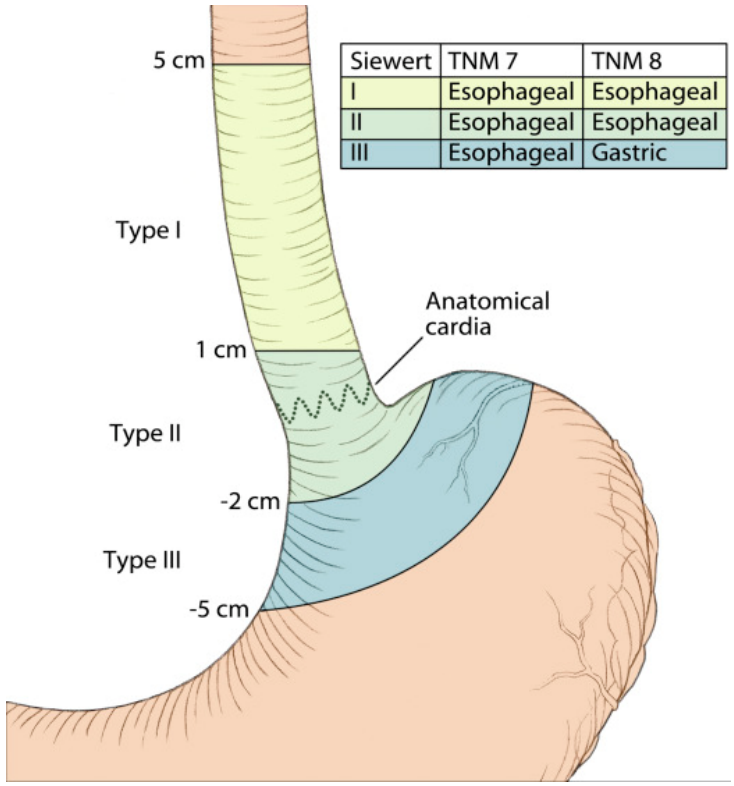


Figure 6.
The Siewert classification of tumors arising in the EG junction according to TNM 7 and TNM 8, respectively. Reprinted from Surgical Clinics, Mazer *et al*. (115) with permission from Elsevier.

Table 1.
TNM classification 8th edition.

Esophageal (and EG junction Siewert type I-II) cancer	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ / high-grade dysplasia
T1	Tumor invades lamina propria, muscularis mucosae, or submucosa
T1a	Tumor invades lamina propria or muscularis mucosae
T1b	Tumor invades submocosa
T2	Tumor invades muscularis propria
T3	Tumor invades adventitia
T4	Tumor invades adjacent structures
T4a	Tumor invades pleura, pericardium, azygos vein, diaphragm or peritoneum
T4b	Tumor invades other adjacent structures such as aorta, vertebral body or trachea
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastais
N1	Metastasis in 1 or 2 regional lymph nodes
N2	Metastasis in 3 to 6 regional lymph nodes
N3	Metastasis in ≥ 7 regional lymph nodes
M0	No distant metastasis
M1	Distant metastasis

Gastric (and EG junction Siewert type III) cancer	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ: intraepithelial tumor without invasion of the lamina propria, high-grade dysplasia
T1	Tumor invades lamina propria, muscularis mucosae, or submucosa
T1a	Tumor invades lamina propria or muscularis mucosae
T1b	Tumor invades submocosa
T2	Tumor invades muscularis propria
T3	Tumor invades subserosa
T4	Tumor perforates serosa (visceral peritoenum) or invades adjacent structures
T4a	Tumor perforates serosa
T4b	Tumor invades adjacent structures (spleen, transverse colon, liver, diaphragm, pancreas, abdominal wall, adrenal gland, kidney, small intestine, or retroperitoneum)
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastais
N1	Metastasis in 1 or 2 regional lymph nodes
N2	Metastasis in 3 to 6 regional lymph nodes
N3	Metastasis in ≥ 7 regional lymph nodes
M0	No distant metastasis
M1	Distant metastasis

Treatment of localized disease

Gastric and esophageal cancer

Initially, both esophageal and gastric cancer were treated with surgery alone. However, in 2001 the randomized INT 0116 study was published, demonstrating a prolonged overall survival (OS) in patients resected for gastric cancer and EG junction adenocarcinoma who in addition to surgery received adjuvant chemoradiotherapy (CRT) (fluorouracil + 45 Gy). This American study concluded that adjuvant CRT should be considered for patients with high risk of recurrence, and the treatment strategy was implemented in the United States, although in Europe the study was criticized for the limited lymph node dissection performed. Of the included patients, the majority (54%) had undergone D0 dissection (i.e. incomplete dissection of perigastric lymph nodes) and solely 10% had undergone a formal D2 dissection (i.e. resection of perigastric lymph nodes and additional removal of nodes along the left gastric, the common hepatic, the splenic and the left hepatoduodenal artery) (116).

In 2006, the UK MAGIC trial was published, wherein patients resected for adenocarcinoma in the stomach, EG junction or distal esophagus were randomized to perioperative ECF (epirubicin, cisplatin and fluorouracil) or surgery alone. The results showed a 5-year OS benefit of 36% vs 23% for the perioperative subgroup, leading to a rapid implementation of perioperative treatment also in many European countries. Although, in Sweden the chemotherapy triplet regimen was modified to EOX (epirubicin, oxaliplatin and capecitabine), due to a more convenient administration setup and the fact that the REAL2 study published 2008 showed the EOX triplet to be superior to ECF in the metastatic setting (117).

Today, the most commonly used perioperative chemotherapy combination for gastric and EG junction adenocarcinoma is FLOT (fluorouracil, leucovorin, oxaliplatin and docetaxel). This is based on a German randomized trial presented in 2017, comparing the FLOT regimen to an ECF/ECX regimen. The results showed a 5-year OS survival benefit of 45% vs 36% in favor of FLOT (118).

However, only 15-20% of the patients benefit from the addition of chemotherapy to surgery alone (118-120) and, additionally, solely half of the patients are able to complete the postoperative component in a perioperative treatment setting (118, 119). Considering the fact that preoperative treatment is more tolerable than postoperative treatment, the Australian trial TOP GEAR is investigating whether preoperative chemoradiotherapy is superior to chemotherapy in gastric adenocarcinoma (including EG junction tumors Siewert type I and II). The study is still ongoing, however an interim analysis has demonstrated preoperative chemoradiotherapy to be equally safe and feasible compared to chemotherapy, without any additional adverse effect on surgical morbidity (121).

The optimal adjuvant treatment has also been investigated in the Dutch CRITICS trial wherein patients with gastric or EG junction adenocarcinoma were randomized to either adjuvant chemoradiotherapy or chemotherapy, preceded by neoadjuvant chemotherapy (NAC) and surgery for both subgroups. The trial was published in 2018 and showed no significant difference in 5-year OS for either subgroup (122). However, in a *post-hoc* analysis published in 2021, including only the patients proceeding to adjuvant treatment, the adjuvant chemotherapy group had a better 5-year OS (123).

Of note, there is a variability in treatment approach for locally advanced gastric cancer in Asia compared to the Western world. In Asia, surgery up-front followed by adjuvant chemotherapy is considered the standard treatment course. Two of the studies supporting this approach for gastric cancer is the Asian CLASSIC trial (capecitabine and oxaliplatin vs surgery alone) and the ACTS trial (S1; tegafur/gimeracil/oteracil vs surgery alone), both demonstrating adjuvant chemotherapy to be superior (124, 125). Furthermore, the JACCRO GC-07 study demonstrated a significantly better 3-year relapse-free survival when intensifying the adjuvant treatment by adding docetaxel to S1(126). In Western populations, there are no larger studies comparing the efficacy of adjuvant vs perioperative chemotherapy, however a meta-analysis including smaller trials have provided some supporting evidence for adjuvant chemotherapy being superior to surgery alone (127).

For patients with locally advanced esophageal cancer, the randomized Dutch CROSS trial, published in 2012, has been pivotal (128). The CROSS study showed a significantly better OS for neoadjuvant CRT (paclitaxel, carboplatin + 41.4 Gy) compared to surgery alone, hence CRT was introduced as the new standard treatment. The patients included in the CROSS trial had tumors located either in the esophagus or in the EG junction, of which 75% were adenocarcinomas and 23% were SCC (128). The long term results demonstrated a 5-year OS benefit of 47% vs 33% for the CRT treated group, however the SCC subgroup had the greatest benefit (129).

Whether adenocarcinomas in the esophagus are best treated with neoadjuvant CRT or chemotherapy only is still unresolved. The Neo-AEGIS trial investigated neoadjuvant chemoradiotherapy according to CROSS vs perioperative chemotherapy according to modified MAGIC or FLOT for esophageal and EG junction adenocarcinomas, however the trial was closed early since no evidence of the former being superior to the latter could be identified (130). There are further ongoing trials but no results are to date published (131, 132). Studies supporting treatment with chemotherapy are e.g. the aforementioned MAGIC and FLOT4 studies, since distal esophageal tumors and EG junction tumors were included in those trials (118, 119). This was also the case for the French FFCD-9703 trial on resectable EG adenocarcinoma, wherein 75% of the included patients had tumors located in the distal esophagus or in the EG junction. This study demonstrated

perioperative chemotherapy (cisplatine and fluorouracil) to be superior to surgery alone (120).

Lastly, definitive chemoradiotherapy (i.e. radiotherapy to a higher dose and no planned surgical treatment) is another treatment option for esophageal cancer, however furthermost for SCC, and, hence, this will not be further discussed in this thesis.

In conclusion, the current European treatment recommendations for fit patients with locally advanced esophageal adenocarcinoma is perioperative chemotherapy using the FLOT regimen or neoadjuvant chemoradiotherapy according to the CROSS protocol. For esophageal SCC, chemoradiotherapy is recommended either neoadjuvantly or as definitive treatment. For gastric cancer, the standard treatment is perioperative chemotherapy using the FLOT regimen.

Prognostic and predictive factors

To enable personalized treatment of patients there is a great need to identify prognostic as well as predictive factors. According to a meta-analysis by van den Ende *et al*., based on curative randomized clinical trials, 16 prognostic factors were identified for esophageal cancer and 23 for gastric cancer, e.g. age, resection radicality, TNM categories, differentiation grade, nutritional status and comorbidities (133). In the clinic today, TNM classification is the most important tool available for prognostication (133). An improvement of note is the current 8th TNM edition, wherein separate classifications for cTNM, pTNM and ypTNM have been implemented, the latter referring to pathological classification after neoadjuvant treatment with chemotherapy or chemoradiotherapy. According to results from the previously mentioned MAGIC trial, histopathologic response was beneficial for prognosis in the pretreated subgroup, however not independently of nodal status, which was the only independent predictor of improved OS (119). Furthermore, in a pooled international analysis of gastric cancer, EBV-positivity is suggested as a beneficial prognostic factor for gastric cancer (134) and moreover proposed to have a more likely response to immune checkpoint inhibition (135, 136).

As aforementioned, solely 15-20% of patients receiving perioperative chemotherapy actually benefit from this additional treatment (118-120). Unfortunately, there are to date no markers to single out which patients to treat. However, a *post-hoc* analysis of the MAGIC study demonstrated that patients with MSI-High (MSI-H)/deficient mismatch repair (dMMR) tumors had significantly worse survival than patients with microsatellite stable (MSS)/proficient mismatch repair (pMMR) tumors when treated with chemotherapy in addition to surgery, thus suggesting perioperative chemotherapy to be unbeneficial for patients with MSI-

H/dMMR tumors (137). Furthermore, in a *post-hoc* analysis of the CLASSIC trial, no significant benefit was identified for patients with MSI-H tumors when treated with adjuvant chemotherapy compared to surgery alone, whereas patients with MSS tumors had a significant better disease-free survival when treated with adjuvant chemotherapy. In addition, when stratifying the MSS group into PD-L1 positive and PD-L1 negative tumors, only the group with MSS and PD-L1 immune cell (PD-$L1_{IC}$) negativity had better prognosis when treated with chemotherapy. In the entire cohort, regardless of adjuvant treatment, both MSI-H tumors and PD-$L1_{IC}$ positivity were independent prognostic factors of prolonged survival, furthermore, MSI-H was related to a favorable prognosis regardless of PD-$L1_{IC}$ positivity. Based on these results, the authors suggest that patients with MSS and PD-$L1_{IC}$ negative gastric tumors should be treated with adjuvant chemotherapy, while patients with MSI-H tumors, regardless of PD-L1 expression, should be spared from additional treatment (138).

Another study demonstrated stage III gastric cancer patients with dMMR tumors to have a better prognosis than patients with pMMR tumors, regardless of treatment with adjuvant chemotherapy (139). Moreover, a study on early gastric cancer could not find any association with MSI status and survival (140).

Taken together, MSI or dMMR gastric tumors have been proposed to be insensitive to chemotherapy, and furthermore indicative of a good prognosis. However, given the low incidence of MSI tumors in gastric (8-10%) and esophageal (5%) adenocarcinomas, the number of analyzed cases are few, and the debate regarding the benefit of perioperative treatment for this patient group is still ongoing (141, 142). In Sweden, however, the forthcoming national guidelines will recommend up-front analysis of MSI/MMR status for gastric adenocarcinomas, and for patients with MSI-H/dMMR tumors, surgery only should be considered.

A predictive biomarker in routine use is human epidermal growth factor receptor 2 (HER2), that predicts the benefit of HER2 targeted therapies in the palliative setting (143). Furthermore, MMR/MSI status and PD-L1 expression are to some extent used to identify patients more likely to respond to immune checkpoint inhibition (144).

The prognostic role of tumor infiltrating immune cells has, as previously mentioned, been investigated in several studies, but their predictive role is less studied. Jiang *et al.* did however conclude that immune cell signatures may be an important tool for prediction to adjuvant chemotherapy in gastric cancer (145).

Targeted therapies

A targeted therapy aims at blocking specific mechanisms in the tumor cell to prevent growth and spread of the disease. The first therapy to be approved for patients with HER2 positive gastric or EG junction adenocarcinoma was the monoclonal antibody trastuzumab. The approval was based on the ToGA trial wherein addition of trastuzumab to fluoropyrimidine and cisplatin as first line palliative treatment increased OS with several months, especially for patients with tumors showing high HER2 expression (i.e. immunohistochemistry (IHC) 2+ and flouresence in situ hybridization (FISH) positive or IHC 3+) (143). Moreover, the benefit of anti-HER2 therapies (pertuzumab and trastuzumab) in addition to perioperative chemotherapy in gastric and EG junction adenocarcinoma is under present investigation in the yet unpublished EORTC1203 INNOVATION study (146). However, in the randomized phase 2 PETRARCA study on HER2 positive resectable EG adenocarcinoma the addition of trastuzumab and pertuzumab to perioperative FLOT improved the rate of complete histopathologic response but with no significant difference in disease-free survival and at the price of higher toxicity (147).

Furthermore, an Asian randomized phase 2 study has shown the antibody drug conjugate trastuzumab deruxtecan (cytotoxic topoisomerase I inhibitor), given in the third line or later, to significantly improve response and OS compared to chemotherapy alone in gastric and EG junction adenocarcinoma (148). The therapy was approved by the Food and Drug Administration (FDA) in January 2021 for patients with locally advanced or metastatic gastric or EG junction adenocarcinoma priorly treated with a trastuzumab-based regimen (149).

The second targeted therapy to be approved for gastric and EG junction adenocarcinomas was ramucirumab, a monoclonal antibody targeting the vascular endothelial growth factor receptor 2 (VEGFR2). This therapy has been shown to render an increased median OS of 2.2 months when added to paclitaxel compared to paclitaxel alone in the second line (150). Ramucirumab has also been shown to be efficient as monotherapy after progression on first line therapy compared to placebo (151).

Immune checkpoint inhibitors

Ipilimumab, a monoclonal antibody targeting CTLA-4, was the first immune checkpoint inhibitor (ICI) to be approved by the FDA in 2011 for treatment of metastatic melanoma. Since then, significant progress has been made, and as of December 2020, six other different ICIs have been approved across 20 different tumor entities and two tissue agnostic conditions (MSI/dMMR tumors and tumor mutational burden high cancers) (144, 152). Notably, despite the rapid expansion of

immune checkpoint inhibition in general, the approvals for EG cancer have been limited, especially in earlier lines of therapy (153, 154), and treatment with ICI has only recently been sanctioned as a treatment option for EG adenocarcinoma in Europe. The latest approvals will be highlighted in the following section.

In 2021, the PD-1 inhibitor nivolumab was FDA and European Medicines Agency (EMA) approved in the first line setting, combined with chemotherapy (fluoropyrimidine and platinum), for patients with unresectable gastric, EG junction or esophageal adenocarcinoma (HER2 negative) with a PD-L1 combined positive score (CPS) ≥ 5. The CPS is the total number of PD-L1 stained tumor cells, lymphocytes and macrophages divided by the total number of viable tumor cells, multiplied by 100. The approval is based on the global CheckMate-649 trial (155) wherein patients were randomized to either nivolumab and chemotherapy (fluoropyrimidine and oxaliplatin) or nivolumab and ipilimumab or chemotherapy alone, regardless of PD-L1 expression. The primary endpoint was OS and progression free survival for patients with a PD-L1 CPS ≥ 5. The nivolumab plus ipilimumab arm was closed early due to futility. The results showed nivolumab in combination with chemotherapy to be superior to chemotherapy alone (median OS was 14.4 months in the nivolumab-containing arm and 11.1 months in the chemotherapy arm) (155).

Moreover, as a result of the KEYNOTE-590 trial (156), the PD-1 inhibitor pembrolizumab was FDA and EMA approved (also in Sweden) in 2021 in combination with chemotherapy (fluoropyrimidine and platinum) as first line treatment for patients with locally advanced unresectable, or metastatic esophageal carcinoma, or HER2-negative EG junction (Siewert type I) adenocarcinoma, with PD-L1 positivity (CPS ≥ 10).

Lastly, in 2021, as a result of the CheckMate-577 trial (157), the PD-1 inhibitor nivolumab was FDA and EMA approved, regardless of PD-L1 status, as adjuvant treatment for patients with radically resected esophageal or EG junction cancer after neoadjuvant chemoradiotherapy without complete histopathological response in the resected primary tumor specimen (157).

There are several ongoing trials in EG adenocarcinoma investigating ICI as monotherapy or in combinations with chemotherapy, targeted therapies or doublet ICI, in the perioperative, adjuvant as well as palliative setting (153, 158, 159).

Aims of the thesis

Overarching aim

The overarching aim of this thesis was to map the tumor immune microenvironment and identify immune markers that might improve prognostication and response prediction in EG adenocarcinoma.

Specific aims

- To examine the intercorrelation and prognostic impact of different immune markers in chemoradiotherapy-naïve tumors.
- To examine the density and intercorrelations of different immune markers in diagnostic biopsy specimens before NAC and in resected primary tumor specimens after NAC.
- To examine the relationship of different immune markers in diagnostic biopsy specimens before NAC with histopathological response.
- To examine the potential effect of NAC on the prognostic value of different immune markers.

Methods and patients

Tissue microarray and immunohistochemistry

Principles

Tissue microarray (TMA) is a high-throughput technique for analyzing protein expression in tissues. This method was introduced in 1998 by Kononen *et al.* (160) and enables rapid analysis across many tumor samples simultaneously. Moreover, this technique solely needs a fraction of antibody and tissue material compared to analysis of full-face tissue sections, thus conserving both reagents and valuable tissue material (161). As illustrated in Figure 7, TMAs are constructed by arraying representative cylindrical core biopsies (0.6-2 millimeters in diameter) from formalin fixed paraffin embedded (FFPE) donor blocks into a recipient paraffin block. The recipient block is then sliced into thin (in general 4 μm) sections that are mounted on microscope glass slides, allowing for detection of proteins by IHC, or of DNA or mRNA by in situ hybridization (160).

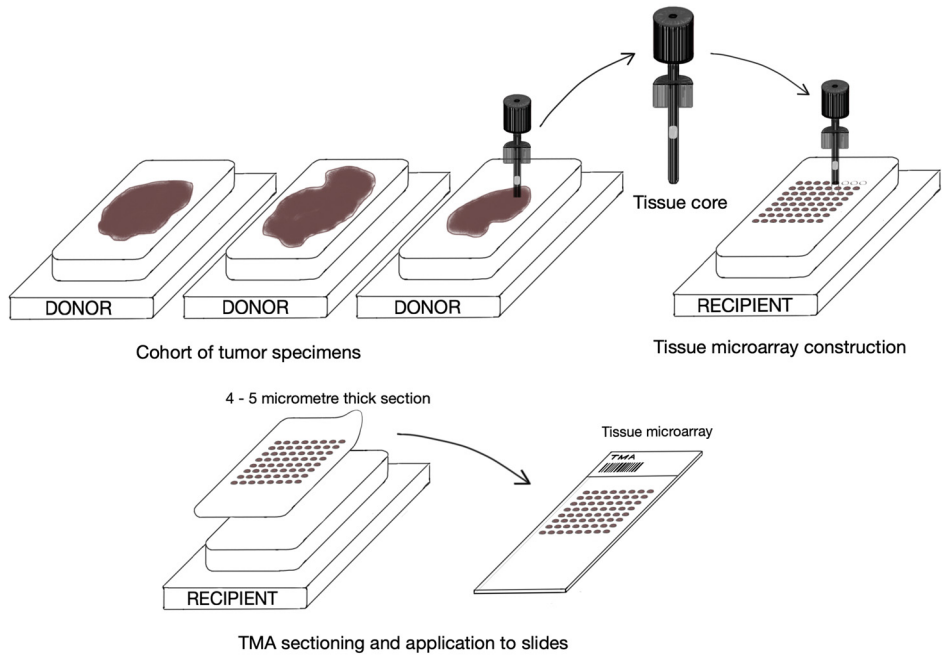


Figure 7.
Construction of a tissue microarray. Cylindrical tissue core biopsies are collected from donor blocks, arrayed into a recipient block, sliced into thin sections and mounted on a microscope glass slide. Reprinted courtesy of Dr Gustav Andersson.

The widely used IHC technique was conceptualized and introduced by Albert H. Coons and colleagues in the 1940s. IHC enables visualization (both expression and location) of proteins or other molecules in tissue samples by the use of antibodies (162). The technique has since then been further developed, and in the 1960s Nakane *et al*. introduced enzyme conjugated antibodies, thereby making detection in a light microscope possible (163). When tumor tissue has been retrieved through biopsy or resection it promptly needs to be fixated in order to prevent autolysis. The most commonly used agent for this purpose is formalin (164). Formalin binds to proteins and crosslinks them to methylene bridges, thereby stabilizing the tissue. The formalin fixed tissue is then dehydrated and embedded in paraffin resulting in FFPE blocks. The sequence of slide preparation is then usually as follows; The formalin fixed tissue sample is pretreated with heat and an antigen retrieval solution with the aim to break the crosslinks formed by the formalin fixation. After this, a primary antibody solution is applied to the glass slide which reacts with the tissue antigen through binding to an epitope. The last step is to add a secondary antibody labeled with an enzyme, e.g. peroxidase, which enables detection in a light microscope.

Antibodies can either be monoclonal or polyclonal. Polyclonal antibodies have a higher detection sensitivity due to their ability to recognize multiple epitopes of the same antigen, while monoclonal antibodies have a higher specificity since they are developed to only recognize one antigen epitope (164). To ensure accurate results, antibodies need to be validated both regarding their specificity and sensitivity (164).

Methodological considerations

The issue of tumor heterogeneity may be a possible concern when utilizing the TMA technique. Hence, to reduce the risk of sampling bias, it is recommended to obtain multiple tissue cores from different sites of each tumor, i.e. central as well as peripheral areas, and when possible also from different tumor blocks from the same patient (161). This has been done whenever feasible for the TMAs utilized in the papers included in the present thesis. Furthermore, the TMA technique has been demonstrated to provide equal prognostic information as compared to full-face tissue sections (161). Besides tumor heterogeneity there is also the risk of reaction bias (e.g. tissue processing and antigen retrieval) and interpretation bias (e.g. types and clones of antibodies utilized, inter- and intra-observer variability) (164). To reduce these risks, the same experienced research engineer has constructed all TMAs and performed the majority of stainings for all papers (I-IV) included in the present thesis. Furthermore, all manual assessments have been carried out by two independent observers, one of whom was the same board-certified pathologist (KJ). Regarding the issue of immune marker heterogeneity there are some studies demonstrating PD-L1 and PD-1 expression in TMAs to be quite comparable to full-face tissue sections (165, 166). Furthermore, the papers in the present thesis investigating PD-L1 expression utilized a platform-independent clone shown to be comparable to 3 of the FDA approved clones used in the clinic (167). Moreover, a study on gastric cancer, utilizing the same antibody clone, investigated the representability of PD-L1 expression on surface biopsies compared to the expression in whole surgical resection specimens in chemoradiotherapy-naïve tumors. This study concluded that the accuracy of assessment of PD-L1 status is equal in endoscopic biopsies and resected tumor specimens, provided that the mean value of at least 4 biopsy samples are assessed. Of note, using fewer biopsies resulted in false-negative results (168).

Manual and digital assessment

Principles

The assessment of staining can be done either manually or digitally. In this thesis, digital image analysis (DIA) was used to quantify $CD3^+$ and $CD8^+$ T cells in paper I, whereas the remaining immune marker quantifications were assessed manually.

Manual assessment

Classical manual assessment is a quantitative or semi-quantitative method to estimate staining positivity regarding intensity and/or percentage. Manual assessment comes with some limitations, foremost there is an inter- and intra-observer variation. It can also be challenging to truly quantify a large number of cells. To resolve these issues, and to reduce hands-on time, DIA was developed.

Digital image analysis

The sequence of digital image analysis can be described as follows; The TMA glass slide is scanned in high resolution. Thereafter the image is manually pre-processed by excluding necrotic areas and inadequate cores. An algorithm is then applied with the aim to optimize the quantification of the investigated biomarker. The algorithm takes several factors into account such as staining intensity, threshold and morphology. Although DIA can reduce the intra- and inter-observer variation seen in manual scoring (169, 170), there are other limitations to this method. For example, indistinct cell borders can make it difficult for the DIA to separate and count cells accurately (171), and a variety in the quality as well as intensity of staining can also pose a challenge given that DIA is based on a static algorithm.

Methodological considerations

Although DIA was partly developed to reduce hands-on time, it was quite time-consuming to quantify $CD3^+$ and $CD8^+$ T cells in paper I, and there is still an element of inter- and intra-observer variability. In addition, manual assessment is still the most commonly used technique in the clinic when assessing immune cells, even if digitalization is on the rise. In light of these facts, manual assessment was favored over digital analysis when scoring the majority of immune markers included in the present thesis.

Assessment of immune markers

Principles

The evaluation of immune markers has been described in the material and methods section in all papers included in the present thesis. In Table 2, an overview of the cellular localization of the investigated immune markers is presented. Figure 8, illustrates IHC images of different TAM subsets as well as their infiltration into tumor nests (TN).

Table 2.

Immune marker	Cellular localization
CD3	Membrane, cytoplasm
CD8	Membrane
FoxP3	Nucleus
CD20	Predominately cell membrane with some cytoplasmic staining
IGKC	Membrane
NKp46	Membrane
CD68	Cytoplasm
CD163	Membrane
MARCO	Membrane
PD-L1	Membrane, cytoplasm
PD-1	Membrane

Methodological considerations

Given the general lack of a uniform standard for assessing and scoring immune markers, as well as the advancements in identifying certain immune cell subsets, some methodological features should be highlighted.

Macrophages

When reviewing the literature, the most commonly used marker to estimate total TAM infiltration is CD68, and CD163 is an established M2 marker (23, 172). A study by Jeremiasen *et al*. (20) investigated the prognostic impact of macrophages in cohort I, and when doing so, single IHC was performed with CD68 and CD163, respectively, allowing for the total TAM infiltration to be assessed as well as the $CD163^+$ TAM infiltration. However, as an improvement of the immune cell identification method in paper IV (based on cohort II), double staining with CD68 and CD163 was applied, as shown in Figure 8, with the intention to better distinguish both of the extreme TAM phenotypes (M1 and M2). This is an illustration of methodological evolution, enabling more precise assessments and specific results.

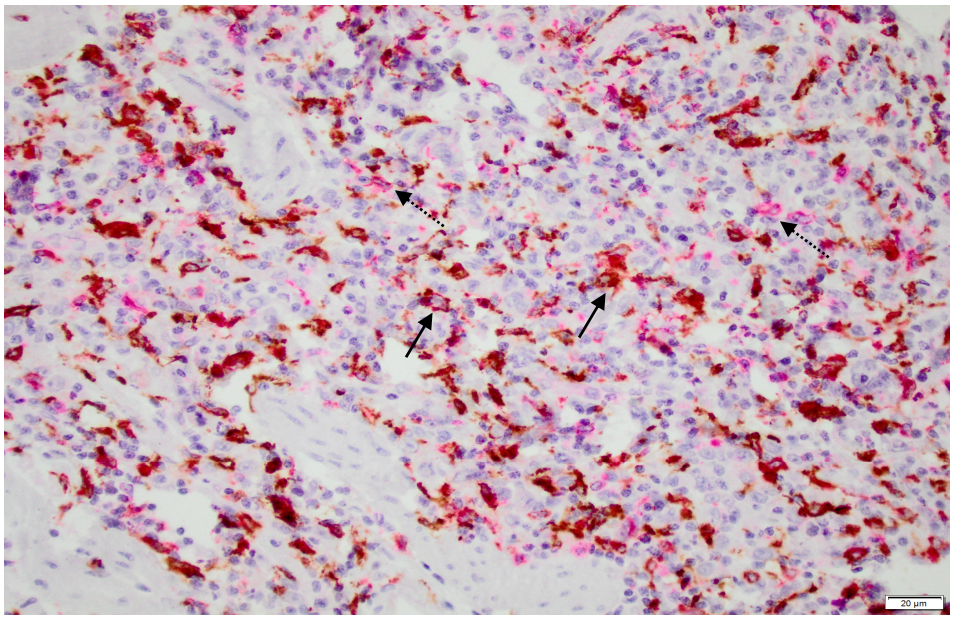


Figure 8.
Sample IHC image of a double staining. $CD68^+/CD163^-$ TAM are indicated by dashed arrows and $CD68^+/CD163^+$ TAM are indicated by solid arrows.

NK and NKT cells

In paper I, NKp46 was utilized as a marker to identify NK cells. NKp46 is a strictly NK specific marker, present in both active and resting cells (173). However, as discussed previously, NKT cells also express the receptor, and, hence, no differentiation could be made between these two immune cells in paper I. To improve the assessment of NK and NKT cells, respectively, double staining with CD3 and CD56 was performed on the pre-treatment biopsies and post-treatment surgical specimens included in cohort II. As CD3 (pan T cell marker) is not expressed on NK cells but solely on T cells and NKT cells, $CD56^+/CD3^-$ immune cells were considered NK cells and $CD56^+/CD3^+$ immune cells were considered NKT cells. Unfortunately, due to an insufficient amount of tissue in many of the pre-treatment biopsies, the analyses of NK and NKT cells were not included in paper IV.

PD-L1

The scoring of PD-L1 is of high relevance today considering the rapid implementation of ICI and the use of PD-L1 as a predictive biomarker for both anti-PD-1 and anti-PD-L1 therapies (168). The heterogeneous expression of PD-L1 has been discussed previously, but the scoring system and cut-off values deserve some additional attention. In paper II, the expression of $PD\text{-}L1^+$ immune cells and tumor

cells, respectively, was denoted in categories of; <10%, 10-49%, 50-100% for PD-$L1_{IC}$ and <1%, 1-4%, 5-9%, 10-49%, 50-100% for PD-$L1^{+}$ tumor cells (PD-$L1_{TC}$), according to a previous study (165). In paper III a continuous score was applied. In both papers, immune cells and tumor cells were annotated separately. Notably, there are different definitions of positive/high vs negative/low PD-L1 cutoffs when reviewing the literature regarding the prognostic value of PD-L1, as is the case for PD-1_{IC} expression, hence making comparisons between studies somewhat difficult. However, most importantly, in clinical trials investigating the predictive value of PD-L1, the CPS is used. The CPS score is divided into different cut-off values, such as 1,5,10. In general, a higher cutoff value reduces the rates of positive cases but increases the likelihood of clinical benefit from immune checkpoint inhibition (168).

Histopathological response

In paper III and IV in the present thesis, histopathological response following neoadjuvant chemotherapy was evaluated in the resected primary tumors. There are different pathological tumor regression grading (TRG) systems in use, however all are based on two main concepts. Either the amount (percentage) of residual tumor cells is estimated, or the relation between residual tumor and regressive fibrosis (descriptive) (174). In the present thesis, the former concept was applied in terms of the TRG system described by Chirieac (175), wherein the histopathological response is divided into four groups, as illustrated in Figure 9.

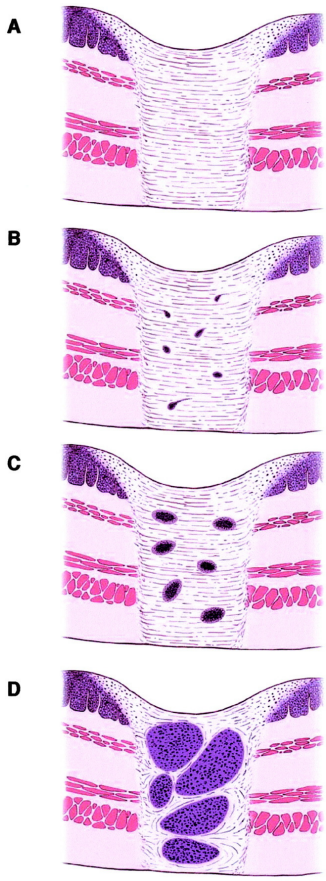


Figure 9.
Illustrattion of the TRG system according to Chirieac; (A) No residual carcinoma, (B) 1–10% residual carcinoma, (C) 11–50% residual carcinoma, (D) Greater than 50% residual carcinoma. Reprinted from Chierieac *et al.* (175) with permission from Wiley.

The Cancer Genome Atlas

TCGA is a project with the aim to catalogue and reveal genome alterations related to cancer through large-scale genome sequencing and multi-dimensional analyses. Initially, the project covered only selected tumors with poor prognosis (brain, lung and ovarian cancer), but since 2009 a total of 30 different tumor types has been added and analyzed, including EG cancer (176). The data are publicly available to support improvement of both the diagnostics, treatment and the prevention of cancer, and TCGA cooperates with institutions both in the USA and Europe. For the second paper included in this thesis, data from TCGA was utilized to investigate the prognostic value of PD-L1 and PD-1 at the transcript level (mRNA).

Patients

Cohort I

This study cohort encompasses a consecutive series of 174 patients, all diagnosed with esophageal or gastric adenocarcinoma. All patients were subjected to surgical resection at Skåne University Hospital, Sweden, between January 1, 2006 and December 31, 2010. No patients received neoadjuvant or perioperative oncological treatment, and solely 13 (7,5%) received adjuvant treatment. Data on survival and recurrence reach until march 2016. Of note, three patients with known cM1 disease were resected with a palliative intent to reduce symptoms (such as bleeding). Furthermore, in 16 patients, M1 disease (such as carcinomatosis or paraaortic lymph node metastases) was revealed either during surgery or in the resected specimens. A summary of the clinical characteristics is presented in Table 3.

Cohort II

This study cohort encompasses a consecutive series of 148 patients, diagnosed with esophageal or gastric adenocarcinoma. All patients received NAC and 78 (66.7%) received adjuvant chemotherapy at Skåne University Hospital Sweden. NAC started between January 1, 2008 and December 31, 2014. After NAC, 118 patients underwent surgical resection. Data on survival status and recurrence reach until December 31, 2017. A summary of the clinical characteristics is presented in Table 4.

Table 3.
Patient and tumor characteristics of all 174 patients included in cohort I.

Factor	Entire cohort n (%)	* (nb)
Age (years)		
Mean	70.2	
Median	70.0	
Range	42.6-94.4	
Sex		
Female	39 (22.4)	
Male	135 (77.6)	
Location		
Esophagus	99 (56.9)	
Stomach	75 (43.1)	
cT stage		
T1	9 (5.2)	
T2	81 (46.6)	
T3	83 (47.7)	
Missing data	1 (0.6)	
cN stage		
N1	117 (67.2)	
N2	42 (24.1)	
N3	14 (8.0)	
Missing data	1 (0.6)	
cM stage		* Paraaortic lymph node metastasis (3), skeletal metastasis (2), subcutaneous metastasis (1)
M	167 (96.0)	
M1*	6 (3.4)	
Missing data	1 (0.6)	
pT stage		
T1	19 (10.9)	
T2	32 (18.4)	
T3	96 (55.2)	
T4	27 (15.5)	
pN stage		
N0	59 (33.9)	
N1	30 (17.2)	
N2	41 (23.6)	
N3	44 (25.3)	
pM stage		
M0	155 (89.1)	
M1	19 (10.9)	
R classification		
R0	119 (68.4)	
R1	46 (26.4)	
R2	9 (5.2)	
Differentiation grade		
Low grade	8 (4.6)	
Intermediate grade	53 (30.5)	
High grade	113 (64.9)	
Lauren classification		
Intestinal	120 (69.0)	
Mixed	9 (5.2)	
Diffuse	45 (25.9)	
Adjuvant treatment		
No	161 (92.5)	
Chemoradiotherapy	11 (6.3)	
Chemotherapy	1 (0.6)	
Radiotherapy	1 (0.6)	

TTR (years)		
Mean	3.0	
Median	1.6	
Range	0.1-9.3	
OS (years)		
Mean	3.4	
Median	2.4	
Range	0.1-9.3	
Recurrence status		
No	67 (38.5)	
Yes	81 (46.6)	
Unknown/Not applicable	26 (14.9)	
Vital status		
Alive	47 (27.0)	
Dead	127 (73.0)	

Table 4.
Patient and tumor characteristics of all 148 patients included in cohort II.

Factor	Entire cohort n (%)	* (nb)
Age (years)		
Mean	63.2	
Median	65.2	
Range	21.1-81.0	
Sex		
Female	58 (39.2)	
Male	90 (60.8)	
Location		
Esophagus	39 (26.4)	
Stomach	109 (73.6)	
cT stage		
T1	1 (0.7)	
T2	54 (36.5)	
T3	87 (58.8)	
T4	6 (4.1)	
cN stage		
N0	80 (54.1)	
N1	50 (33.8)	
N2	14 (9.5)	
N3	4 (2.7)	
cM stage		
M0	136 (91.6)	* Lymph node metastasis (M1 position) (6), Liver metastasis (3), adrenal gland metastasis (1), ovarian metastasis (1), ascites (1)
M1*	12 (8.1)	
Neoadjuvant treatment		
Chemotherapy	148 (100)	
Fluoropyrimidine+platinum ≥ 8 weeks, no irinotecan	121 (81.8)	
Resection		
Yes	118 (79.7)	*Perioperative findings of advanced disease (20), Liver metastases after 1 cycle of chemotherapy (1), Progressive disease (1), Performance status not allowing surgery (3), Death (2), Patient's wish (3)
No*	30 (20.3)	
Adjuvant treatment		
Chemotherapy	78 (66.7)	
Fluoropyrimidine+platinum ≥ 8 weeks, no irinotecan	46 (59.7)	
Chemoradiotherapy	10 (8.5)	
None	29 (24.8)	

Missing data	1	
No resection	30	
Histopathological response (residual cancer cells)		
0%	13 (11.1)	
1-10%	13 (11.1)	
11-50%	46 (39.3)	
>50%	45 (38.5)	
Missing data	1	
No resection	30	
ypT stage		
T0	13 (11.1)	
T1	14 (12.0)	
T2	22 (18.8)	
T3	26 (35.9)	
T4	26 (17.6)	
Missing data	1	
No resection	30	
ypN stage		
N0	52 (44.1)	
N1	28 (23.7)	
N2	18 (15.3)	
N3	20 (16.9)	
No resection	30	
ypM stage		
M0	113 (95.8)	* Lymph node metastasis in M1-position (1), Liver metastasis (1), Ovarian metastasis (1), Peritoneal deposit (2)
M1*	5 (4.2)	
No resection	30	
R classification		
R0	97 (82.2)	
R1	19 (16.1)	
R2	2 (1.7)	
No resection	30	
DIfferentiation grade		
Low grade	3 (2.4)	
Intermediate grade	52 (42.3)	
High grade	68 (55.3)	
Missing data	25	
Lauren classification		
Intestinal	65 (52.8)	
Mixed	16 (13.0)	
Diffuse	42 (28.4)	
Missing data	25	
TTR (years)		
Mean	3.5	
Median	3.0	
Range	0.1-9.4	
OS (years)		
Mean	3.7	
Median	3.3	
Range	0.2-9.4	
Recurrence status		
No	67 (45.3)	
Yes	49 (33.1)	
Unknown/Not applicable	32 (21.6)	
Vital status		
Alive	63 (42.6)	
Dead	85 (57.4)	

Study cohort considerations

The two cohorts are overlapping in time, however cohort I started inclusion 2 years earlier (2006 vs 2008) and ended 4 years earlier (2010 vs 2014). During this time period, the treatment of EG adenocarcinoma has partly changed. The main event was the introduction of neoadjuvant/perioperative treatment for gastric cancer which started in 2007 in Skåne, Sweden (based on the MAGIC trial (119)). Initially, younger patients with risk factors were treated with NAC, however gradually it has become the standard treatment independent of risk factors and age, provided a good performance status and no contraindicating comorbidities. For esophageal adenocarcinomas, neoadjuvant chemoradiotherapy was initiated 2012 (based on the CROSS study (128)). However this treatment has successively changed towards neoadjuvant/perioperative chemotherapy given that adenocarcinomas are less sensitive to radiation than SCC, and that the studies supporting neoadjuvant/perioperative treatment of gastric cancer also included tumors of the distal esophagus (119, 120). The few patients in cohort II who did receive neoadjuvant chemoradiotherapy were excluded.

Cohort II, encompassing both pre-treatment biopsy specimens and post-treatment surgical specimens, is of particular interest since it enables investigation of the effect of NAC on the tumor immune microenvironment. When reviewing the literature, very few studies are based on paired pre-treatment and post-treatment specimens in EG adenocarcinoma, and another strength is the comparatively large number of cases in this cohort. A limitation is however the possible sampling bias when investigating diagnostic biopsies compared to surgical specimens.

Statistical considerations

All statistical methods applied in this thesis have been described in paper I-IV, respectively. Therefore, the following section will mainly focus on statistical considerations not previously addressed.

The general principle of having a certain number of events per predictor variable (EPV) when performing Cox models is based on studies dating back to the 1990´s. These studies demonstrated a risk for e.g. increased bias, variability and unreliable confidence interval (CI) coverage when utilizing less than 10 EPV in a model. However, only the number of events varied when performing these simulated tests (177, 178). Hence, in 2007 another study was carried out, wherein the former constant variables such as sample size and distribution varied. The results indicated that the former rule of 10 could be tranquilized without severe consequences. Specifically, 5-9 EPV could be comparable to 10-16 EPV, while 2-4 EPV may cause more frequent problems. Of course, bigger samples and more events are preferable (179).

In paper I, II and III included in this thesis, the confounding factors adjusted for in the multivariable Cox proportional hazards analyses were selected based on background knowledge (i.e. established prognostic factors). EPV was not primarily accounted for. However, when reflecting on the inability to run multivariable analyses in some categories in paper III (due to a small number of cases and few events), the selection procedure evolved. Thus, in paper IV, EPV was taken into consideration and only established prognostic factors with a significant association to OS in univariable analysis were included in multivariable analyses.

There has also been a variation and stepwise evolution in the choice of method to determine the most appropriate prognostic cut-off point to enable survival analyses in the papers included in this thesis. This is mainly due to the general lack of uniform standards for immune marker cutoffs. Nevertheless, one of the primary goals when dichotomizing data in an exploratory study is to find a cutoff that allows for the detection of potentially important findings while avoiding skewness of the data and statistical errors. In paper I and III, in which the immune markers were assessed continuously, both classification and regression tree (CRT) derived cutoffs and median derived cutoffs were constructed for the survival analyses. One of the disadvantages with CRT derived cutoffs is possible skewness of the data, as was the case for some categories in paper III, and thus enhanced risk of type-I errors. However, the downside with a median derived cutoff is that the method *per se* only takes the median value into account when creating the cutoff. In paper IV, neither CRT nor median derived cutoffs were applied. Instead, continuous variables were used, allowing for the identification of potentially important stepwise patterns.

Of note, when performing numerous statistical analyses there is an increasing risk for type I statistical errors to occur. This can be compensated for with e.g. the Bonferroni adjustment, which is calculated by taking the number of tests and dividing them into the alpha value (i.e. the p-value threshold). However, when doing so, the risk of type II errors increases, and given the exploratory nature of these papers, setting the significance level too high comes with a risk that potentially relevant findings are overlooked.

Lastly, in order to make the cohorts comparable, TTR and OS were defined as time from diagnosis (date of the result of the preoperative biopsy) in cohort I and as time from resection in cohort II. Endpoints for TTR and OS did not differ and were defined as the date of radiologically or biopsy verified recurrent disease and the date of death from any cause, respectively.

Summary of results and discussion

Paper I

Herein, we investigated the clinical impact of tumor-infiltrating T lymphocytes ($CD3^+$, $CD8^+$, $FoxP3^+$) and NK cells ($NKp46^+$) in relation to B lymphocyte ($CD20^+$) and plasma cell ($IGKC^+$) infiltration in a cohort encompassing 174 patients with chemoradiotherapy-naïve EG adenocarcinoma (cohort I). The individual prognostic impact of B lymphocytes and plasma cells had been described in a previously published paper (180).

The results demonstrated an association between high infiltration of any T and NK cell subset investigated and an improved OS, with high $CD8^+$ T cell and NK cell density remaining independent prognostic factors. T cells ($CD8^+$ and $FoxP3^+$) were also independent prognostic factors for a prolonged time to recurrence. Compartmental localization (intra-tumoral, tumor-adjacent or stromal) did not affect the prognostic impact for either of the investigated immune cells. The most noteworthy result in this paper was that the strongest beneficial prognostic impact was seen for high T cell infiltration in combination with high B cell and/or plasma cell infiltration. The findings were most evident in gastric cancer, where significant interactions in relation to OS were seen for $CD3^+$, $CD8^+$ and $FoxP3^+$ cells with $CD20^+$ cells, and for $FoxP3^+$ cells with $IGKC^+$ plasma cells. In esophageal tumors, there was only a significant prognostic interaction between $CD3^+$ and $CD20^+$ cells. In addition, when investigating the intercorrelations between the immune cells in the entire cohort, $CD20^+$ B cells correlated strongly, and $IGKC^+$ plasma cells correlated moderately to strongly, with any T cell subset investigated. The findings were similar when stratifying for tumor location. Taken together, these data suggest, that the antitumoral impact of tumor-infiltrating T cells may be highly dependent on a functional interplay between T cells and B cells or plasma cells.

This was the first study to address the potential prognostic interaction of B cells and T cells in EG adenocarcinoma. Since then, B cells in general and their synergistic effect with T cells in particular, has attracted an increasing interest. In a recent study on metastatic melanoma by Cabrita *et al*., the co-occurrence of tumor-associated $CD8^+$ T cells and $CD20^+$ B cells was demonstrated to be an independent predictor of improved survival. Furthermore, the best survival outcome was demonstrated for patients with tumors presenting a combination of tertiary lymphoid structures (TLS) and $CD8^+$ T cells (181). TLS are structures composed of a T cell zone, B cell

follicles, plasma cells, dendritic cells and high endothelial venules (182). Cabrita *et al*., also demonstrated that TLS may have a key role in sustaining an immune-responsive microenvironment, thus leading to an improved response to immune checkpoint blockade (181). The induction of cytotoxic T cell proliferation by TLS has also been suggested in a study on treatment naïve gastric cancer (183), and the presence of TLS has been shown to be associated with an improved outcome in several tumor entities, including gastric cancer (67, 69, 182, 184).

Paper II

Herein, we investigated the expression of PD-L1 on tumor cells (PD-$L1_{TC}$) and tumor-infiltrating immune cells (PD-$L1_{IC}$), and the expression of PD-1 on tumor infiltrating-immune cells (PD-1_{IC}) in 174 cases of chemoradiotherapy-naïve primary EG adenocarcinoma and paired lymph node metastases (cohort I). Particular attention was given to their relationship with MMR status and prognosis. The presence of EBV-positive tumors was also investigated. In addition, the prognostic value of PD-L1 and PD-1 expression was examined at the mRNA level in 354 cases of gastric cancer and 161 cases of esophageal cancer in TCGA.

The results demonstrate that PD-$L1_{IC}$ expression was higher in lymph node metastases compared to primary tumors, correlated with dMMR status and lower TNM stage, and was an independent factor of prolonged survival in patients with chemoradiotherapy-naïve EG adenocarcinoma. PD-$L1_{TC}$ expression did not differ between primary tumors and lymph node metastases, nor did it confer any prognostic value. Furthermore, higher PD-$L1_{TC}$ expression correlated with dMMR status and higher tumor grade. PD-1 expression did not differ between primary tumors and lymph node metastases, correlated with lower T and N stages, and was a prognostic factor for prolonged survival in unadjusted analysis. All EBV-positive tumors (n=3) were located in the stomach, had higher PD-$L1_{TC}$ expression (≥10%) and were MMR proficient. At the transcript level, only high PD-1 expression in gastric cancer was significantly associated with a prolonged survival.

The results from this study indicate that PD-$L1_{IC}$ expression may be a useful biomarker for identifying patients who could possibly be spared from additional chemotherapy or chemoradiotherapy before curative surgery (i.e. frail/elderly patients). In addition, the results highlight the temporal heterogeneity of PD-L1 expression, which is of importance when considering the use of PD-L1 as a predictive biomarker for immune checkpoint inhibitors. Moreover, all patients with EBV-positive tumors were alive at last follow-up, thus supporting the suggested beneficial association for this subtype of gastric cancer. As mentioned previously, EBV-positive tumors have also been proposed to respond better to ICI (135, 136).

Regarding the mRNA data, PD-1 expression was the only marker being significantly associated with prolonged survival, and this was only evident in gastric cancer, not esophageal cancer. Of note, the esophageal cancers in TCGA include both SCC and adenocarcinomas, which might well cloud the results. An overarching issue related to studies on esophageal cancer in general is that the cohorts often contain a mixture of these two histological subtypes, thus making interpretation of data on prognosis and response prediction challenging. Furthermore, at the transcript level, PD-L1 expression did not confer any prognostic value, while our data demonstrated PD-$L1_{IC}$ expression to be a favorable prognostic factor. This probably reflects the fact that the mRNA expression levels represent both immune cells and tumor cells. Therefore, IHC should be the preferable method when assessing PD-L1 expression for the purpose of prognostication or response prediction.

Paper III and Paper IV

In these two papers, we investigated the effect of neoadjuvant chemotherapy (NAC) on the composition of T cells, B cells, PD-L1 expression and different TAM subsets, in a cohort encompassing patients with resected EG adenocarcinoma, all of whom received NAC (cohort II). To this end, the different immune markers were examined regarding their total density as well as their prognostic impact in paired pre-treatment biopsies, post-treatment resected primary tumors and lymph node metastases. The impact of compartmental localization of the immune cells was also investigated.

In paper III, the density and prognostic impact of tumor-infiltrating T cells ($CD8^+$ and $FoxP3^+$), B cells ($CD20^+$), and PD-L1 expression was examined. The results demonstrate an increased density of $CD8^+$ T cells and a decreased density of $FoxP3^+$ T cells and $CD20^+$ B cells in post-NAC specimens, whereas PD-L1 expression was not altered following NAC. No significant associations were found between immune marker density pre-NAC and histopathological response. In pre-NAC specimens, high $FoxP3^+$ density and high PD-$L1_{IC}$ expression were favorable prognostic factors, whereas high $CD8^+$ density was an unfavorable prognostic factor. Neither PD-$L1_{TC}$ expression nor B cell density conferred any prognostic information. In sharp contrast to the pre-NAC situation, high $FoxP3^+$ density post-NAC was an unfavorable prognostic factor. Furthermore, high PD-$L1_{TC}$ expression was associated with a shorter survival. $CD8^+$ T cells, B cells and PD-$L1_{IC}$ expression were not prognostic. Lastly, neither the infiltration of T cells into TN nor the presence of B cell aggregates were prognostic in pre-NAC or post-NAC specimens.

In addition to the diverging prognostic associations for various immune cell subsets pre-NAC and post-NAC, no potential synergistic prognostic effect between B and

T cells was identified in paper III, neither before nor after treatment with NAC. In light of these findings, it appears evident that NAC complicates the picture when it comes to immune cells and prognostication. A potential sampling bias might however also have affected the results, given the varying representativity of the diagnostic biopsies.

Moreover, no prognostic impact of B cell aggregates was identified. However, the presence of TLS *per se* was not investigated, which would have required e.g. a double IHC staining to identify the co-localization of the B and T cells. To the best of our knowledge, only one former study has investigated the additive effect of B and T cells in gastric cancer, and the analyses were only performed on post-NAC specimens. The results demonstrated that the combination of high density of peritumoral $CD20^+$ B cell aggregates and high infiltration of $Tbet^+$ cells in the tumor stroma was a beneficial prognostic factor, regardless of NAC (67).

In paper IV, the infiltration and prognostic impact of $CD68^+/CD163^-$, $CD68^+/CD163^+$ and $MARCO^+$ TAM was investigated. The results demonstrate an increased density of $CD68^+/CD163^+$ TAM and a decreased density of $MARCO^+$ TAM in post-NAC specimens. $CD68^+/CD163^-$ TAM density was not altered following NAC. No prognostic impact could be identified for either TAM subset in pre-NAC specimens regarding total infiltration, however high $CD68^+/CD163^+$ TAM infiltration into TN was an independent unfavorable prognostic factor for TTR. Moreover, in post-NAC specimens, higher total infiltration of $CD68^+/CD163^-$ TAM, as well as the infiltration into TN, were adverse prognostic factors, and, in addition, this was the only TAM subset associated with an established unfavorable prognostic factor (high tumor grade). $MARCO^+$ TAM was not prognostic post-NAC, regardless of compartmental localization. No significant association was found between TAM density in pre-NAC specimens and histopathological regression.

In a previous paper by Jeremiasen *et al.* (20), based on cohort I, increased infiltration of $CD68^+$ and $CD163^+$ TAM, furthermost $CD68^+$ TAM, into TN, was significantly associated with a stepwise reduced survival (20). Similar associations were seen in pre-NAC biopsies in paper IV, more specifically for $CD68^+/CD163^+$ TAM. However, contrastingly, after treatment, high infiltration of $CD68^+/CD163^-$ TAM into TN was an adverse prognostic factor, whereas high infiltration of $CD68^+/CD163^+$ TAM was not. As previously discussed, Jeremiasen *et al*., performed single IHC staining, not allowing for assessment of $CD68^+/CD163^-$ TAM. Thus, the results in these papers are not entirely comparable. Of note, it is also somewhat difficult to make comparisons between chemotherapy-naïve tumors and pre-NAC specimens. Along this line, it would have been of value to compare diagnostic biopsies and surgical specimens in cohort I.

TAM have emerged as a potential target for ICI. However given the plasticity of this highly abundant immune cell subset, both TAM depleting and altering therapies have been suggested (185). In a study by Harada *et al*. on gastric cancer, PD-L1

expression on tumor cells (immune cells were not investigated) was found to be significantly associated with $CD163^+$ macrophage infiltration (186). This finding led the authors to suggest that $CD163^+$ macrophages might promote PD-L1 expression on tumor cells, therefore being a potential target for ICI. In the study by Jeremiasen *et al*., based on cohort I, significant associations were also identified between $CD163^+$ TAM infiltration and $PD\text{-}L1_{TC}$ expression.

The results in paper III and IV further support that chemotherapeutic agents have the ability to alter the immune cell composition in EG adenocarcinoma, although a potential sampling bias must be kept in mind. Altered patterns of immune cell infiltration following NAC have also been demonstrated in other tumor entities such as head and neck squamous cell carcinoma, breast cancer and ovarian cancer (187-189). However, in line with the present results, $CD8^+$ T cells have chiefly been demonstrated to be recruited (187, 190-193) and $FoxP3^+$ T cells have been shown to decrease (194), but also to remain unaltered (187, 191). While neither $PD\text{-}L1_{IC}$ or $PD\text{-}L1_{TC}$ expression was altered upon treatment with NAC in the present investigation, other studies have demonstrated ambiguous results with both decreasing and increasing expression (187, 188, 190, 191, 195). This is also the case for B cells, although these have been much less investigated (189, 192). Lastly, in line with the present results, $CD163^+$ TAM density has been demonstrated to increase following NAC(193).

Conclusions

- The prognostic impact of tumor-infiltrating T cells may be highly dependent on their functional interplay with B cells in chemoradiotherapy-naïve EG adenocarcinoma.
- PD-L1 expression on immune cells, but not on tumor cells, is a prognostic marker in chemoradiotherapy-naïve EG adenocarcinoma.
- The potential effect of NAC should be taken into account when using PD-L1 expression as a predictive marker for selection of patients with EG adenocarcinoma for treatment with immune checkpoint inhibition.
- The composition of the tumor immune microenvironment in pre-treatment biopsies does not predict histopathological response in EG adenocarcinoma.
- Neoadjuvant chemotherapy appears to have the capability to alter the composition and prognostic impact of the tumor immune microenvironment in EG adenocarcinoma.

Future perspectives

The findings in this thesis highlight the need for further studies on the effect of NAC on the tumor immune microenvironment in EG adenocarcinoma, not least in the context of immune checkpoint inhibition, which is given alone or in combination with chemotherapy in different clinical settings today. Such studies should also be extended to include esophageal SCC, and potential differences between the histological types should be considered. Furthermore, given the emerging role of TLS in immune-oncology, it would be of particular interest to evaluate the possible effect of NAC on these structures in paired pre- and post-treatment specimens.

Moreover, despite the increasing use of NAC, it would still also be of value to identify reliable prognostic immune signatures for the identification of patients who could be spared treatment, e.g. those with comorbidities. In this context, a combined score of T cells and B cells, or PD-L1 expression on immune cells, as identified in this thesis, would merit further validation.

Populärvetenskaplig sammanfattning

Cancer är en lömsk sjukdom som har drabbat oss sedan urminnes tider, den äldsta beskrivningen sträcker sig så långt bakåt i tiden som 1500 år före Kristus. Sedan dess har förekomsten av cancer ökat i hela världen på grund av faktorer som en allt längre livslängd, växande befolkning och bättre diagnostik. Cancer uppstår när normala celler i kroppen börjar dela sig ohämmat utan att omgivande celler lyckas bromsa eller stoppa dem. Kroppens eget immunförsvar har visat sig spela en stor roll i kampen mot cancer.

Immunsystemet består huvudsakligen av två delar, det medfödda, ospecifika immunförsvaret, vilket inte behöver någon aktivering utan direkt kan försvara oss mot cancerceller, samt det förvärvade, specifika immunförsvaret som behöver aktiveras innan det kan agera. Fördelen med det specifika immunförsvaret är att det, trots en lite långsammare startsträcka, har förmågan att känna igen specifika strukturer på tumörceller samt minnas dem inför framtida attacker.

Det ospecifika immunförsvaret består av flera olika komponenter, både kroppsliga barriärer (t.ex. hud och slemhinnor), kemiska barriärer (t.ex. magsyra och gallsyra) samt immunceller (tex. makrofager och naturliga mördarceller). Det specifika immunförsvaret består av immunceller (T och B celler) samt antikroppar. Det ospecifika och specifika immunförsvaret samarbetar och kompletterar varandra i kampen mot cancer. Följande är exempel på några viktiga immunförsvarsceller.

Ospecifika immunförsvarsceller

- Makrofager; äter upp cancerceller och motverkar tumörutveckling genom att främja andra immunceller, men under vissa omständigheter kan de även bidra till tumörutveckling genom att hämma andra immunceller.
- Naturliga mördarceller; kan eliminera cancerceller samt aktivera andra immunförsvarsceller.
- Naturliga mördar-T-celler; kan utplåna cancerceller, men framför allt har de immunmodulerande egenskaper. Liknar både mördar-T-celler samt naturliga mördarceller och betraktas därför ligga i gråzonen mellan det ospecifika och specifika immunförsvaret.

Specifika immunförsvarsceller

- Mördar-T-celler; utplånar cancerceller, beskrivs som målinriktade missiler i immunförsvaret mot cancer.
- T hjälparceller; främjar mördar-T-cellernas samt B-cellernas funktion.
- Regulatoriska-T celler; bromsar immunförsvaret för att motverka en alltför stark reaktion.
- Minnes-T-celler; skapar det immunologiska minnet.
- B-celler; producerar antikroppar som märker ut cancerceller så de kan utplånas av andra celler i immunförsvaret.
- Plasmaceller; högspecialiserade B-celler som producerar stora mängder antikroppar.

År 2020 fanns över 1,7 miljoner nya fall av matstrups- och magsäckscancer globalt vilket tillsammans gör dem till de 4:e vanligaste cancerformerna och de näst vanligaste orsakerna till cancerrelaterad död i världen. Prognosen för dessa tumörsjukdomar är dyster vid spridd sjukdom då patienterna i genomsnitt lever mindre än ett år. I Sverige är dessa cancersjukdomar mindre vanliga men mellan åren 2013-2017 insjuknade trots allt 1200 personer årligen.

Cancer i matstrupen förekommer i två olika former som utgår från olika vävnader; skivepitel och adenocarcinom. Skivepitelcancer dominerar globalt sett och stod 2018 för 85% av alla ny fall medan adenocarcinom stod för resterande 15%. I västvärlden har man dock över de senaste 30 åren sett en drastisk ökning av typen adenocarcinom som nu till och med är vanligare än skivepitelcancer.

Globalt sett har insjuknandet och dödligheten i magsäckscancer sjunkit under de senaste 50 åren i princip hela världen. Detta beror på att ”magsårsbakterien” *Helicobacter Pylori*, vilken är den största kända riskfaktorn för magsäckscancer, nu kan upptäckas och behandlas bort med antibiotika. Övriga kända riskfaktorer för magsäckscancer är rökning samt lågt intag av frukt och grönsaker samt högt intag av salt, inlagd mat och alkohol. Riskfaktorer för att utveckla cancer i matstrupen av adenocarcinomtyp är framför allt sura uppstötningar och fetma medan det för skivepiteltyp framför allt är rökning och alkohol.

Det vanligaste tidiga symtomet på cancer i matstrupen och magsäcken är svårigheter att svälja. Övriga symtom kan vara tex sura uppstötningar, illamående och ofrivillig viktnedgång. Då symtomen ofta är diffusa, framförallt vid magsäckscancer, är dessa cancrar ofta svåra att upptäcka, men vid misstanke om cancer skall man utredas med gastroskopi där man via en liten kamera tittar ner i matstrupen och magsäcken och tar vävnadsprov, så kallade biopsier, ifall man ser

cancermisstänkta förändringar. Om vävnadsproverna påvisar cancer skall man utredas vidare med en skiktröntgen av bröstkorgen och buken för att utesluta att cancersjukdomen har spridit sig (metastaserat) till andra organ i kroppen. För patienter med lokal sjukdom utan spridning av tumören finns möjlighet till bot via kirurgi men trots att man opererar bort all synlig cancer är det endast 20-25% av alla patienter som lever efter 5 år. Behandlingen har dock utvecklats och genom tillägg av cellgifter och i vissa fall strålning har överlevnaden förbättrats.

Vid matstrups- och magsäckscancer av adenocarcinomtyp är standardbehandlingen att ge cellgiftsbehandling både före och efter operation, vilket har visat sig öka 5-års överlevnaden till ca 45%. För patienter som inte kan opereras på grund av spridning av tumören till andra organ, eller för att de inte bedöms klara av en operation, erbjuds behandling i bromsande och symtomlindrande syfte. Målet med den behandlingen är att förlänga livet, samt att förbättra livskvaliteten för patienten, men med tanke på att patienter med spridd sjukdom i genomsnitt endast lever 1 år trots olika former av uppbromsande behandling är livskvalitet av högsta vikt. Det finns sålunda ett stort behov att förbättra behandlingen och prognosen för patienter med matstrups- och magsäckscancer, både för de med botbar och för de med icke botbar sjukdom.

Forskning kring immunceller, dess funktion samt interaktion med tumörceller pågår intensivt sedan länge och detta forskningsområde benämns ”immunonkologi”. Immunförsvarets celler kan infiltrera tumörens mikromiljö, dvs den miljö som omger cancercellerna. Tumörmikromiljön består även av bland annat stödjeceller och signalerande molekyler. En av de viktigaste mekanismerna bakom cancerutveckling är tumörcellernas förmåga att undgå igenkänning och utrotning av tumörinfiltrerande immunceller. En av de regulatoriska vägar som tumörcellerna använder sig av för att hämma immunförsvaret är att binda in till så kallade ”checkpoints”, dvs hämmande receptorer som uttrycks av flera olika typer av immunceller. I normala fall binder immuncellerna själva in till dessa receptorer, om en viss tillfällig dämpning av immunförsvaret är nödvändig, men tumörcellerna kan också utnyttja receptorerna till att blockera immunförsvaret totalt vilket leder till utebliven eliminering av tumörcellerna. Ett av det mest spännande exemplen inom immunonkologi är framtagandet av antikroppar, så kallad immunterapi. Antikropparna blockerar tumörcellerna från att binda in till checkpointreceptorerna och därmed kan immuncellerna fortsätta sitt jobb med att utrota tumörcellerna. För vissa cancerdiagnoser har detta inneburit banbrytande resultat, med dramatiskt förbättrad överlevnad, men för matstrups- och magsäckscancer har utvecklingen gått tämligen långsamt. Det behövs även bättre metoder för att identifiera vilka tumörer som är känsliga för denna typ av behandling.

Det är också av största vikt att ta reda på vilka patienter som har nytta av sedvanlig cellgiftsbehandling, då detta tillsammans med strålbehandling fortsatt utgör basen för den mediciniska behandlingen för patienter med matstrups- och magsäckscancer.

Mot denna bakgrund har syftet med mitt avhandlingsarbete varit att analysera den inflammatoriska tumörmikromiljön vid cancer i matstrupe och magsäck. Dels för att kartlägga hur cellgifter påverkar immuncellernas sammansättning och dels för att undersöka immuncellernas prognostiska värde.

Min avhandling omfattar fyra stycken delarbeten baserade på två olika patientgrupper. Tumörvävnad studerades dels från 174 patienter med adenocarcinom i matstrupe eller magsäck som opererades utan att erhålla förbehandling med cellgifter, dels från 148 patienter med adenocarcinom i matstrupe eller magsäck som fick förbehandling med cellgifter. I den senare patientgruppen analyserades såväl diagnostiska tumörvävnadsprov (biopsier) före behandlingsstart som vävnadsmaterial från de bortopererade tumörerna. För att studera immuncellerna i tumörmikromiljön har vävnadssnitten färgats in med olika antikroppar, varefter mängden fastställts med ljusmikroskop samt i enstaka fall även med digital bildanalys. Vi har sedan undersökt om mängden immunceller, enskilt eller i kombinationer, kan ge information om risken för återfall av cancer och död.

I delarbete I, som är baserat på den första patientgruppen, analyserades olika typer av T-celler (mördar-T-celler, och regulatoriska- T celler), naturliga mördar celler samt deras relation till B-celler och plasmaceller. Våra resultat visade att hög tumörinfiltration av alla olika T celler och naturliga mördarceller var gynnsamma för patientöverlevnad. Det största nyhetsvärdet i det här arbetet var att kombinationen av hög tumörinfiltration av T celler samt B och/eller plasmaceller innebar den absolut mest gynnsamma prognosen. När artikeln publicerades fanns inga studier som visat detta i matstrups- och magsäckscancer, men därefter har B-celler fått allt mer uppmärksamhet även inom andra tumörtyper.

I delarbete II, som också är baserat på den första patientgruppen, studerades uttrycket av checkpointproteinet programmed death-1 (PD-1), som finns på olika immunceller, samt dess mottagarmolekyl (ligand) PD-L1, som uttrycks både på olika immunceller och tumörceller, i vävnadsprov både från såväl tumörer som närliggande lymfkörtlar. Vi undersökte även förekomsten av Epstein-Barr virus (EBV)-infekterade tumörer. Man vet sedan tidigare att det prognostiska värdet av PD-L1 och PD-1 i magsäcks- och matstrupscancer är väldigt varierande samt att EBV-infekterade tumörer troligen har bättre prognos. Våra resultat visade att uttrycket av PD-L1 var högre i lymfkörtelmetastaser än i modertumörer. Högt PD-L1 uttryck på tumörinfiltrerande immunceller var den starkaste gynnsamma faktorn för patientöverlevnad. Högt PD-1 uttryck på immunceller var också till viss del en gynnsam faktor för överlevnad men inte lika tydligt som PD-L1. Vidare fann vi tre fall av magsäckscancer som var positiva för EBV och alla dessa patienter hade lång överlevnad. Det viktigaste fyndet i det här arbetet var att PD-L1 uttrycket skiljde sig mellan tumör och lymfkörtel, vilket indikerar att biopsier bör tas från flera lokaler om man vill undersöka PD-L1 uttryck. Detta är högst aktuellt idag då PD-L1 används som en markör för vilka patienter som skall erbjudas immunterapi. Ett högt PD-L1 uttryck på immunceller, vilket indikerade en gynnsam prognos, skulle också

kunna hjälpa till att bespara sköra patienter onkologisk tilläggsbehandling före botande kirurgi.

I delarbete III och IV, som baserades på den andra patientgruppen, studerades olika typer av T-celler (mördar-T-celler och regulatoriska T celler), B-celler, uttrycket av PD-L1 på immunceller respektive tumörceller, samt infiltrationen av olika makrofager i både diagnostiska vävnadsprov tagna före operation samt vävnadsprov från tumören efter operation. I förekommande fall undersöktes även närliggande lymfkörtelmetastaser.

I delarbete III visade våra resultat att andelen mördar- T-celler ökade medan andelen regulatoriska T celler och B-celler minskade efter cellgiftsbehandling. PD-L1 uttrycket påverkades inte. Före cellgiftsbehandling var en hög infiltration av regulatoriska T celler gynnsamt för överlevnad, men efter behandling var det tvärtemot ogynnsamt. Förekomst av mördar-T-celler var ogynnsamt för överlevnad före cellgiftsbehandling men hade inget samband med överlevnad efter cellgiftsbehandling. Andelen B-celler hade ingen påverkan på överlevnaden vare sig före eller efter cellgiftsbehandling. Högt PD-L1 uttryck på immunceller, men inte på tumörceller, var gynnsamt för överlevnad före cellgiftsbehandling medan högt PD-L1 uttryck på tumörceller, men inte immunceller, var förknippat med sämre överlevnad efter cellgiftsbehandling. Slutligen fann vi ingen koppling mellan immuncellsinfiltration i vävnadsproverna före cellgiftsbehandling och hur mycket tumören krympte efter cellgiftsbehandlingen.

I delarbete IV visade våra resultat att även infiltrationen av makrofager påverkas av cellgiftsbehandling. Här kunde man se att patientöverlevnaden ändrades beroende på om makrofagerna befann sig intill tumörcellerna eller inte. Sammanfattningsvis var det sämre för överlevnaden att ha en hög infiltration intill tumörcellerna i vävnadsproverna före cellgiftsbehandling av den makrofagtyp som generellt sett anses vara ogynnsam för överlevnad. Däremot var det prognostiskt ogynnsamt att efter cellgiftsbehandling ha en hög total såväl som tumörcellsnära infiltration i vävnadsproverna av den makrofagtyp som generellt sett anses vara gynnsam för överlevnad. Inte heller för makrofager kunde vi se något samband mellan infiltration i vävnadsprovet före cellgiftsbehandling och hur mycket tumören krympte efter cellgiftsbehandlingen.

Sammanfattningsvis var resultaten både i delarbete III och IV komplexa, då det prognostiska värdet av olika immuncellstyper skilde sig före och efter cellgiftsbehandling. Resultaten är betydelsefulla, då de belyser hur cellgiftsbehandling tycks kunna påverka sammansättningen av olika typer av immunceller och möjligen också deras påverkan på tumörutvecklingen. Det behövs därför mer forskning kring hur immunförsvaret förändras av cellgiftsbehandling, för att få en djupare förståelse av mekanismer som kan driva eller hämma cancerutveckling, samt hur dessa påverkas av behandling.

Acknowledgements

I would like to express my deepest gratitude to all of you who have supported me through this work, with a special thanks to the following:

Karin Jirström, main supervisor. Thank you so much for sharing your extraordinary enthusiasm for research in particular, and life in general. It has been an adventure to be part of your research group! I am so grateful for all the opportunities I have been given, and all the knowledge I have acquired.

Björn Nodin, co-supervisor. Thank you so much for always believing in me, making me smile, and putting your trust in me with packing your luggage before check-in. And of course, for magnificent work in the lab!

Karin Leandersson, co-supervisor. Thank you for excellent advice in the field of immunology, it has been invaluable to me.

Claes Hjalmarsson, co-supervisor, Head of the Department of Surgery, Halland Hospital, and awesome lead-guitarist. Thank you so much for our scientific discussions, your magnificent general guidance and for bringing rock n´roll to me!

David Borg, although not my formal co-supervisor, you have always taken the time for "exciting" discussions, both regarding research and patients. Your knowledge and advice have been invaluable to me. I also want to thank you for teasing me whenever needed (don´t stop)!

Former and present fellow PhD students in the Jirström group; Gustav Andersson, Jonna Berntsson, Jacob Elebro, Richard Fristedt, Karolina Boman, Margareta Heby, Charlotta Hedner, Anna Larsson, Sebastian Lundgren, Sofie Olsson Hau, Alexandra Petersson, Bahar Rastegar, Emelie Carnevi, Christina Siesing, Maja Svensson, Sara Wahlin and Noori Zenderokh thank you all for fun times, great scientific discussions and memorable moments all over the world.

Co-authors not previously mentioned, Carl-Fredrik Warfvinge, Jakob Eberhard, Patrick Micke, Cheng Zhang, Mathias Uhlén, Adil Mardinoglu, Albin Lindén, Jakob Nygaard, thank you for exciting collaborations.

Susanne André and Magnus Zätterström for fantastic administrative support.

Former and present Head of Department of Clinical Sciences, Lund, Bo Baldetorp and Mikael Bodelsson, for creating a stimulating research environment.

The Department of Oncology, Lund, thank you all for making me feel like a part of your clinic since day one. It has been truly rewarding working with you. A special thanks to the colleagues in the GI oncology team for always supporting me.

All my wonderful colleagues at the Department of Surgery, Halland Hospital, including nurses, assistant nurses and secretaries. You are all truly amazing and incredibly hard working, you make the hospital world go round! A special thanks to my closest co-workers at the Department of Oncology.

Karin Porsmyr and Hanna Svensson, thank you both for all your help with administration, general understanding and support!

Lotta Lundgren, thank you for all your support and all the good times we have had sharing apartments, laughing and working together. I really admire your spirit and broad knowledge in the field of oncology!

Ilva Bostedt, thank you for never-ending generosity and for giving me a home away from home. You are a truly amazing person and oncologist and you have inspired me in so many ways.

Lars Åhlund, thank you for your mentorship from the very beginning of my career, I look forward to many more years of guidance and golf.

My late grandparents Doris and Eric Lindahl, you have meant so much to me. I miss you both dearly.

My mother, Christina Lindahl, thank you for being the most wonderful mother imaginable and for always believing in me. Your support and love mean the world to me. I love you!

My uncle, Sven Lindahl, thank you for providing laughter, music, and delicious dinners. You bring so much joy to my life!

My brother Dennis Svensson, thank you for all your support and for being such a good listener during this journey. You always brighten my mood and I'm so grateful to be included in your life.

My twin brother Marcus Svensson Linnman, how lucky I am to have spent my childhood with you. More good times ahead!

Malin Grönberg, you are the most wonderful friend possible. You have supported me whenever needed, thank you also for letting me be part of your family, I am so thankful for having you in my life.

Johanna Wikland, you are an amazing friend and a true inspiration! Thank you for endless calls from the other side of the world, always supporting and fortune telling whenever I need it the most.

Eva Moraeus, thank you for being a companion in surf and life.

Alexander Klun, thank you for creating an awesome front page and all fun times we have had in the snow.

To all my dear friends on and off the water, thank you for bringing good vibes and lots of cheers!

To my former, present and future patients, as well as all patients participating in studies, you are my everyday heroes and make my working days meaningful.

WBTMSMFEAEWYWHYNSHMTWTMLL

References

1. Faguet GB. A brief history of cancer: age-old milestones underlying our current knowledge database. Int J Cancer. 2015;136(9):2022-36.
2. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100(1):57-70.
3. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.
4. Gasteiger G, D'Osualdo A, Schubert DA, Weber A, Bruscia EM, Hartl D. Cellular Innate Immunity: An Old Game with New Players. J Innate Immun. 2017;9(2):111-25.
5. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. Nat Rev Cancer. 2006;6(1):24-37.
6. Underhill DM, Gordon S, Imhof BA, Nunez G, Bousso P. Elie Metchnikoff (1845-1916): celebrating 100 years of cellular immunology and beyond. Nat Rev Immunol. 2016;16(10):651-6.
7. Munro DAD, Hughes J. The Origins and Functions of Tissue-Resident Macrophages in Kidney Development. Front Physiol. 2017;8:837.
8. Watanabe S, Alexander M, Misharin AV, Budinger GRS. The role of macrophages in the resolution of inflammation. J Clin Invest. 2019;129(7):2619-28.
9. Li Q, Barres BA. Microglia and macrophages in brain homeostasis and disease. Nat Rev Immunol. 2018;18(4):225-42.
10. Misharin AV, Morales-Nebreda L, Reyfman PA, Cuda CM, Walter JM, McQuattie-Pimentel AC, et al. Monocyte-derived alveolar macrophages drive lung fibrosis and persist in the lung over the life span. J Exp Med. 2017;214(8):2387-404.
11. Kielbassa K, Vegna S, Ramirez C, Akkari L. Understanding the Origin and Diversity of Macrophages to Tailor Their Targeting in Solid Cancers. Front Immunol. 2019;10:2215.
12. Nielsen SR, Schmid MC. Macrophages as Key Drivers of Cancer Progression and Metastasis. Mediators Inflamm. 2017;2017:9624760.
13. Biswas SK, Allavena P, Mantovani A. Tumor-associated macrophages: functional diversity, clinical significance, and open questions. Semin Immunopathol. 2013;35(5):585-600.
14. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity. 2014;41(1):14-20.
15. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol. 2010;11(10):889-96.

16. Bogels M, Braster R, Nijland PG, Gul N, van de Luijtgaarden W, Fijneman RJ, et al. Carcinoma origin dictates differential skewing of monocyte function. Oncoimmunology. 2012;1(6):798-809.
17. Jing J, Yang IV, Hui L, Patel JA, Evans CM, Prikeris R, et al. Role of macrophage receptor with collagenous structure in innate immune tolerance. J Immunol. 2013;190(12):6360-7.
18. Sun H, Song J, Weng C, Xu J, Huang M, Huang Q, et al. Association of decreased expression of the macrophage scavenger receptor MARCO with tumor progression and poor prognosis in human hepatocellular carcinoma. J Gastroenterol Hepatol. 2017;32(5):1107-14.
19. Lundgren S, Karnevi E, Elebro J, Nodin B, Karlsson MCI, Eberhard J, et al. The clinical importance of tumour-infiltrating macrophages and dendritic cells in periampullary adenocarcinoma differs by morphological subtype. J Transl Med. 2017;15(1):152.
20. Jeremiasen M, Borg D, Hedner C, Svensson M, Nodin B, Leandersson K, et al. Tumor-Associated CD68(+), CD163(+), and MARCO(+) Macrophages as Prognostic Biomarkers in Patients With Treatment-Naive Gastroesophageal Adenocarcinoma. Front Oncol. 2020;10:534761.
21. Jensen TO, Schmidt H, Moller HJ, Hoyer M, Maniecki MB, Sjoegren P, et al. Macrophage markers in serum and tumor have prognostic impact in American Joint Committee on Cancer stage I/II melanoma. J Clin Oncol. 2009;27(20):3330-7.
22. Medrek C, Ponten F, Jirstrom K, Leandersson K. The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients. BMC Cancer. 2012;12:306.
23. Wang XL, Jiang JT, Wu CP. Prognostic significance of tumor-associated macrophage infiltration in gastric cancer: a meta-analysis. Genet Mol Res. 2016;15(4).
24. Cao W, Peters JH, Nieman D, Sharma M, Watson T, Yu J. Macrophage subtype predicts lymph node metastasis in oesophageal adenocarcinoma and promotes cancer cell invasion in vitro. Br J Cancer. 2015;113(5):738-46.
25. Forssell J, Oberg A, Henriksson ML, Stenling R, Jung A, Palmqvist R. High macrophage infiltration along the tumor front correlates with improved survival in colon cancer. Clin Cancer Res. 2007;13(5):1472-9.
26. Trinchieri G. Biology of natural killer cells. Adv Immunol. 1989;47:187-376.
27. Long EO, Kim HS, Liu D, Peterson ME, Rajagopalan S. Controlling natural killer cell responses: integration of signals for activation and inhibition. Annu Rev Immunol. 2013;31:227-58.
28. Carbone E, Neri P, Mesuraca M, Fulciniti MT, Otsuki T, Pende D, et al. HLA class I, NKG2D, and natural cytotoxicity receptors regulate multiple myeloma cell recognition by natural killer cells. Blood. 2005;105(1):251-8.
29. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol. 2008;9(5):503-10.
30. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. Immunol Today. 1990;11(7):237-44.

31. Smyth MJ, Hayakawa Y, Takeda K, Yagita H. New aspects of natural-killer-cell surveillance and therapy of cancer. Nat Rev Cancer. 2002;2(11):850-61.
32. Zhang S, Liu W, Hu B, Wang P, Lv X, Chen S, et al. Prognostic Significance of Tumor-Infiltrating Natural Killer Cells in Solid Tumors: A Systematic Review and Meta-Analysis. Front Immunol. 2020;11:1242.
33. Ishigami S, Natsugoe S, Tokuda K, Nakajo A, Che X, Iwashige H, et al. Prognostic value of intratumoral natural killer cells in gastric carcinoma. Cancer. 2000;88(3):577-83.
34. Svensson MC, Warfvinge CF, Fristedt R, Hedner C, Borg D, Eberhard J, et al. The integrative clinical impact of tumor-infiltrating T lymphocytes and NK cells in relation to B lymphocyte and plasma cell density in esophageal and gastric adenocarcinoma. Oncotarget. 2017;8(42):72108-26.
35. Yang C, Cheng H, Zhang Y, Fan K, Luo G, Fan Z, et al. Anergic natural killer cells educated by tumor cells are associated with a poor prognosis in patients with advanced pancreatic ductal adenocarcinoma. Cancer Immunol Immunother. 2018;67(12):1815-23.
36. McEwen-Smith RM, Salio M, Cerundolo V. The regulatory role of invariant NKT cells in tumor immunity. Cancer Immunol Res. 2015;3(5):425-35.
37. Montoya CJ, Pollard D, Martinson J, Kumari K, Wasserfall C, Mulder CB, et al. Characterization of human invariant natural killer T subsets in health and disease using a novel invariant natural killer T cell-clonotypic monoclonal antibody, 6B11. Immunology. 2007;122(1):1-14.
38. Peng LS, Mao FY, Zhao YL, Wang TT, Chen N, Zhang JY, et al. Altered phenotypic and functional characteristics of CD3+CD56+ NKT-like cells in human gastric cancer. Oncotarget. 2016;7(34):55222-30.
39. Arasanz H, Gato-Canas M, Zuazo M, Ibanez-Vea M, Breckpot K, Kochan G, et al. PD1 signal transduction pathways in T cells. Oncotarget. 2017;8(31):51936-45.
40. Cavanagh M FE. T-cell activation https://www.immunology.org/public-information/bitesized-immunology/systems-and-processes/t-cell-activation: Brittish Society for immunology; [
41. Abbas AK LA, Pillai S. Basic immunology: Functions and disorders of the immune system. Fourth edition ed: ELSEVIER SAUNDERS.
42. Borst J, Ahrends T, Babala N, Melief CJM, Kastenmuller W. CD4(+) T cell help in cancer immunology and immunotherapy. Nat Rev Immunol. 2018;18(10):635-47.
43. Laidlaw BJ, Craft JE, Kaech SM. The multifaceted role of CD4(+) T cells in CD8(+) T cell memory. Nat Rev Immunol. 2016;16(2):102-11.
44. Raskov H, Orhan A, Christensen JP, Gogenur I. Cytotoxic CD8(+) T cells in cancer and cancer immunotherapy. Br J Cancer. 2021;124(2):359-67.
45. Grywalska E, Pasiarski M, Gozdz S, Rolinski J. Immune-checkpoint inhibitors for combating T-cell dysfunction in cancer. Onco Targets Ther. 2018;11:6505-24.
46. Crespo J, Sun H, Welling TH, Tian Z, Zou W. T cell anergy, exhaustion, senescence, and stemness in the tumor microenvironment. Curr Opin Immunol. 2013;25(2):214-21.

47. Nishikawa H, Sakaguchi S. Regulatory T cells in tumor immunity. Int J Cancer. 2010;127(4):759-67.
48. Lee JS, Won HS, Sun S, Hong JH, Ko YH. Prognostic role of tumor-infiltrating lymphocytes in gastric cancer: A systematic review and meta-analysis. Medicine (Baltimore). 2018;97(32):e11769.
49. Shen Z, Zhou S, Wang Y, Li RL, Zhong C, Liang C, et al. Higher intratumoral infiltrated Foxp3+ Treg numbers and Foxp3+/CD8+ ratio are associated with adverse prognosis in resectable gastric cancer. J Cancer Res Clin Oncol. 2010;136(10):1585-95.
50. Stein AV, Dislich B, Blank A, Guldener L, Kroll D, Seiler CA, et al. High intratumoural but not peritumoural inflammatory host response is associated with better prognosis in primary resected oesophageal adenocarcinomas. Pathology. 2017;49(1):30-7.
51. Hou J, Yu Z, Xiang R, Li C, Wang L, Chen S, et al. Correlation between infiltration of FOXP3+ regulatory T cells and expression of B7-H1 in the tumor tissues of gastric cancer. Exp Mol Pathol. 2014;96(3):284-91.
52. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol. 2008;26:677-704.
53. Patsoukis N, Bardhan K, Chatterjee P, Sari D, Liu B, Bell LN, et al. PD-1 alters T-cell metabolic reprogramming by inhibiting glycolysis and promoting lipolysis and fatty acid oxidation. Nat Commun. 2015;6:6692.
54. Wilke CM, Wei S, Wang L, Kryczek I, Kao J, Zou W. Dual biological effects of the cytokines interleukin-10 and interferon-gamma. Cancer Immunol Immunother. 2011;60(11):1529-41.
55. Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, et al. Expression of programmed death 1 ligands by murine T cells and APC. J Immunol. 2002;169(10):5538-45.
56. Svensson MC, Borg D, Zhang C, Hedner C, Nodin B, Uhlen M, et al. Expression of PD-L1 and PD-1 in Chemoradiotherapy-Naive Esophageal and Gastric Adenocarcinoma: Relationship With Mismatch Repair Status and Survival. Front Oncol. 2019;9:136.
57. Boger C, Behrens HM, Mathiak M, Kruger S, Kalthoff H, Rocken C. PD-L1 is an independent prognostic predictor in gastric cancer of Western patients. Oncotarget. 2016;7(17):24269-83.
58. Dislich B, Stein A, Seiler CA, Kroll D, Berezowska S, Zlobec I, et al. Expression patterns of programmed death-ligand 1 in esophageal adenocarcinomas: comparison between primary tumors and metastases. Cancer Immunol Immunother. 2017;66(6):777-86.
59. Kollmann D, Ignatova D, Jedamzik J, Chang YT, Jomrich G, Baierl A, et al. PD-L1 expression is an independent predictor of favorable outcome in patients with localized esophageal adenocarcinoma. Oncoimmunology. 2018;7(6):e1435226.
60. Christina Svensson M, Linden A, Nygaard J, Borg D, Hedner C, Nodin B, et al. T cells, B cells, and PD-L1 expression in esophageal and gastric adenocarcinoma

before and after neoadjuvant chemotherapy: relationship with histopathological response and survival. Oncoimmunology. 2021;10(1):1921443.

61. Gao Y, Li S, Xu D, Chen S, Cai Y, Jiang W, et al. Prognostic value of programmed death-1, programmed death-ligand 1, programmed death-ligand 2 expression, and CD8(+) T cell density in primary tumors and metastatic lymph nodes from patients with stage T1-4N+M0 gastric adenocarcinoma. Chin J Cancer. 2017;36(1):61.
62. Mingari MC, Gerosa F, Carra G, Accolla RS, Moretta A, Zubler RH, et al. Human interleukin-2 promotes proliferation of activated B cells via surface receptors similar to those of activated T cells. Nature. 1984;312(5995):641-3.
63. Yuseff MI, Pierobon P, Reversat A, Lennon-Dumenil AM. How B cells capture, process and present antigens: a crucial role for cell polarity. Nat Rev Immunol. 2013;13(7):475-86.
64. Harwood NE, Batista FD. Early events in B cell activation. Annu Rev Immunol. 2010;28:185-210.
65. Kim SS, Sumner WA, Miyauchi S, Cohen EEW, Califano JA, Sharabi AB. Role of B Cells in Responses to Checkpoint Blockade Immunotherapy and Overall Survival of Cancer Patients. Clin Cancer Res. 2021.
66. Wouters MCA, Nelson BH. Prognostic Significance of Tumor-Infiltrating B Cells and Plasma Cells in Human Cancer. Clin Cancer Res. 2018;24(24):6125-35.
67. Hennequin A, Derangere V, Boidot R, Apetoh L, Vincent J, Orry D, et al. Tumor infiltration by Tbet+ effector T cells and CD20+ B cells is associated with survival in gastric cancer patients. Oncoimmunology. 2016;5(2):e1054598.
68. Sakimura C, Tanaka H, Okuno T, Hiramatsu S, Muguruma K, Hirakawa K, et al. B cells in tertiary lymphoid structures are associated with favorable prognosis in gastric cancer. J Surg Res. 2017;215:74-82.
69. Li Q, Zhang D, He W, Chen T, Yan Z, Gao X, et al. CD8(+) T cells located in tertiary lymphoid structures are associated with improved prognosis in patients with gastric cancer. Oncol Lett. 2020;20(3):2655-64.
70. Mauri C, Bosma A. Immune regulatory function of B cells. Annu Rev Immunol. 2012;30:221-41.
71. Shang J, Zha H, Sun Y. Phenotypes, Functions, and Clinical Relevance of Regulatory B Cells in Cancer. Front Immunol. 2020;11:582657.
72. Ni Z, Xing D, Zhang T, Ding N, Xiang D, Zhao Z, et al. Tumor-infiltrating B cell is associated with the control of progression of gastric cancer. Immunol Res. 2020.
73. Ferlay J EM, Lam F, Colombet M, Mery L, Piñeros M, Znaor A, Soerjomataram I, Bray F (2020). Global Cancer Observatory: Cancer Today. Lyon, France: International Agency for Research on Cancer. Available from: https://gco.iarc.fr/today, accessed 14 Oct 2020 [
74. Edgren G, Adami HO, Weiderpass E, Nyren O. A global assessment of the oesophageal adenocarcinoma epidemic. Gut. 2013;62(10):1406-14.
75. Arnold M, Soerjomataram I, Ferlay J, Forman D. Global incidence of oesophageal cancer by histological subtype in 2012. Gut. 2015;64(3):381-7.

76. Arnold M, Ferlay J, van Berge Henegouwen MI, Soerjomataram I. Global burden of oesophageal and gastric cancer by histology and subsite in 2018. Gut. 2020;69(9):1564-71.
77. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021;71(3):209-49.
78. Warren JR, Marshall B. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet. 1983;1(8336):1273-5.
79. Plummer M, Franceschi S, Vignat J, Forman D, de Martel C. Global burden of gastric cancer attributable to Helicobacter pylori. Int J Cancer. 2015;136(2):487-90.
80. Van Cutsem E, Sagaert X, Topal B, Haustermans K, Prenen H. Gastric cancer. Lancet. 2016;388(10060):2654-64.
81. Rota M, Pelucchi C, Bertuccio P, Matsuo K, Zhang ZF, Ito H, et al. Alcohol consumption and gastric cancer risk-A pooled analysis within the StoP project consortium. Int J Cancer. 2017;141(10):1950-62.
82. Murphy G, Pfeiffer R, Camargo MC, Rabkin CS. Meta-analysis shows that prevalence of Epstein-Barr virus-positive gastric cancer differs based on sex and anatomic location. Gastroenterology. 2009;137(3):824-33.
83. Oliveira C, Pinheiro H, Figueiredo J, Seruca R, Carneiro F. Familial gastric cancer: genetic susceptibility, pathology, and implications for management. Lancet Oncol. 2015;16(2):e60-70.
84. Rubenstein JH, Taylor JB. Meta-analysis: the association of oesophageal adenocarcinoma with symptoms of gastro-oesophageal reflux. Aliment Pharmacol Ther. 2010;32(10):1222-7.
85. Hoyo C, Cook MB, Kamangar F, Freedman ND, Whiteman DC, Bernstein L, et al. Body mass index in relation to oesophageal and oesophagogastric junction adenocarcinomas: a pooled analysis from the International BEACON Consortium. Int J Epidemiol. 2012;41(6):1706-18.
86. Freedman ND, Murray LJ, Kamangar F, Abnet CC, Cook MB, Nyren O, et al. Alcohol intake and risk of oesophageal adenocarcinoma: a pooled analysis from the BEACON Consortium. Gut. 2011;60(8):1029-37.
87. Nie S, Chen T, Yang X, Huai P, Lu M. Association of Helicobacter pylori infection with esophageal adenocarcinoma and squamous cell carcinoma: a meta-analysis. Dis Esophagus. 2014;27(7):645-53.
88. Li B, Jiang G, Zhang G, Xue Q, Zhang H, Wang C, et al. Intake of vegetables and fruit and risk of esophageal adenocarcinoma: a meta-analysis of observational studies. Eur J Nutr. 2014;53(7):1511-21.
89. Xie SH, Lagergren J. Social group disparities in the incidence and prognosis of oesophageal cancer. United European Gastroenterol J. 2018;6(3):343-8.
90. Qu X, Ben Q, Jiang Y. Consumption of red and processed meat and risk for esophageal squamous cell carcinoma based on a meta-analysis. Ann Epidemiol. 2013;23(12):762-70 e1.

91. Uhlenhopp DJ, Then EO, Sunkara T, Gaduputi V. Epidemiology of esophageal cancer: update in global trends, etiology and risk factors. Clin J Gastroenterol. 2020;13(6):1010-21.
92. Siewert JR, Holscher AH, Becker K, Gossner W. [Cardia cancer: attempt at a therapeutically relevant classification]. Chirurg. 1987;58(1):25-32.
93. Jain S, Dhingra S. Pathology of esophageal cancer and Barrett's esophagus. Ann Cardiothorac Surg. 2017;6(2):99-109.
94. Taylor PR, Abnet CC, Dawsey SM. Squamous dysplasia--the precursor lesion for esophageal squamous cell carcinoma. Cancer Epidemiol Biomarkers Prev. 2013;22(4):540-52.
95. van der Kaaij RT, Snaebjornsson P, Voncken FE, van Dieren JM, Jansen EP, Sikorska K, et al. The prognostic and potentially predictive value of the Lauren classification in oesophageal adenocarcinoma. Eur J Cancer. 2017;76:27-35.
96. Nagini S. Carcinoma of the stomach: A review of epidemiology, pathogenesis, molecular genetics and chemoprevention. World J Gastrointest Oncol. 2012;4(7):156-69.
97. Lauren P. The Two Histological Main Types of Gastric Carcinoma: Diffuse and So-Called Intestinal-Type Carcinoma. An Attempt at a Histo-Clinical Classification. Acta Pathol Microbiol Scand. 1965;64:31-49.
98. Jencks DS, Adam JD, Borum ML, Koh JM, Stephen S, Doman DB. Overview of Current Concepts in Gastric Intestinal Metaplasia and Gastric Cancer. Gastroenterol Hepatol (N Y). 2018;14(2):92-101.
99. Parsonnet J, Vandersteen D, Goates J, Sibley RK, Pritikin J, Chang Y. Helicobacter pylori infection in intestinal- and diffuse-type gastric adenocarcinomas. J Natl Cancer Inst. 1991;83(9):640-3.
100. Petrelli F, Berenato R, Turati L, Mennitto A, Steccanella F, Caporale M, et al. Prognostic value of diffuse versus intestinal histotype in patients with gastric cancer: a systematic review and meta-analysis. J Gastrointest Oncol. 2017;8(1):148-63.
101. Qiu MZ, Cai MY, Zhang DS, Wang ZQ, Wang DS, Li YH, et al. Clinicopathological characteristics and prognostic analysis of Lauren classification in gastric adenocarcinoma in China. J Transl Med. 2013;11:58.
102. Henson DE, Dittus C, Younes M, Nguyen H, Albores-Saavedra J. Differential trends in the intestinal and diffuse types of gastric carcinoma in the United States, 1973-2000: increase in the signet ring cell type. Arch Pathol Lab Med. 2004;128(7):765-70.
103. Sun K, Jia K, Lv H, Wang SQ, Wu Y, Lei H, et al. EBV-Positive Gastric Cancer: Current Knowledge and Future Perspectives. Front Oncol. 2020;10:583463.
104. Cancer Genome Atlas Research N. Comprehensive molecular characterization of gastric adenocarcinoma. Nature. 2014;513(7517):202-9.
105. Cancer Genome Atlas Research N, Analysis Working Group: Asan U, Agency BCC, Brigham, Women's H, Broad I, et al. Integrated genomic characterization of oesophageal carcinoma. Nature. 2017;541(7636):169-75.

106. Stapley S, Peters TJ, Neal RD, Rose PW, Walter FM, Hamilton W. The risk of oesophago-gastric cancer in symptomatic patients in primary care: a large case-control study using electronic records. Br J Cancer. 2013;108(1):25-31.
107. Daly JM, Fry WA, Little AG, Winchester DP, McKee RF, Stewart AK, et al. Esophageal cancer: results of an American College of Surgeons Patient Care Evaluation Study. J Am Coll Surg. 2000;190(5):562-72; discussion 72-3.
108. Smithers BM, Fahey PP, Corish T, Gotley DC, Falk GL, Smith GS, et al. Symptoms, investigations and management of patients with cancer of the oesophagus and gastro-oesophageal junction in Australia. Med J Aust. 2010;193(10):572-7.
109. Stephens MR, Lewis WG, White S, Blackshaw GR, Edwards P, Barry JD, et al. Prognostic significance of alarm symptoms in patients with gastric cancer. Br J Surg. 2005;92(7):840-6.
110. Axon A. Symptoms and diagnosis of gastric cancer at early curable stage. Best Pract Res Clin Gastroenterol. 2006;20(4):697-708.
111. Nationellt vårdprogram matstrups- och magsäckscancer. 2019. https://kunskapsbanken.cancercentrum.se/diagnoser/matstrups-och-magsackscancer/vardprogram/2019 [
112. Sobin L GM, Wittekind C. . TNM Classification of Malignant Tumours, 7th Edition. Wiley-Blackwell 2009 [Available from: http://eu.wiley.com/WileyCDA/ WileyTitle/productCd-1444332414.html.
113. Brierley J, Gospodarowicz M, Wittekind C. TNM Classification of Malignant Tumours, 8th Edition. 8th ed: Wiley-Blackwell; 2017 January 2017. 272 p.
114. Rice TW, Ishwaran H, Ferguson MK, Blackstone EH, Goldstraw P. Cancer of the Esophagus and Esophagogastric Junction: An Eighth Edition Staging Primer. J Thorac Oncol. 2017;12(1):36-42.
115. Laura M. Mazer GAP. What Is the Best Operation for Proximal Gastric Cancer and Distal Esophageal Cancer? Volume 99, Jun 1 2019, pages 457-469. Elsevier.
. Surgical Clinics: Elsevier; 2019 Jun 1. 13 p.
116. Macdonald JS, Smalley SR, Benedetti J, Hundahl SA, Estes NC, Stemmermann GN, et al. Chemoradiotherapy after surgery compared with surgery alone for adenocarcinoma of the stomach or gastroesophageal junction. N Engl J Med. 2001;345(10):725-30.
117. Cunningham D, Starling N, Rao S, Iveson T, Nicolson M, Coxon F, et al. Capecitabine and oxaliplatin for advanced esophagogastric cancer. N Engl J Med. 2008;358(1):36-46.
118. Al-Batran SE, Homann N, Pauligk C, Goetze TO, Meiler J, Kasper S, et al. Perioperative chemotherapy with fluorouracil plus leucovorin, oxaliplatin, and docetaxel versus fluorouracil or capecitabine plus cisplatin and epirubicin for locally advanced, resectable gastric or gastro-oesophageal junction adenocarcinoma (FLOT4): a randomised, phase 2/3 trial. Lancet. 2019;393(10184):1948-57.
119. Cunningham D, Allum WH, Stenning SP, Thompson JN, Van de Velde CJ, Nicolson M, et al. Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. N Engl J Med. 2006;355(1):11-20.

120. Ychou M, Boige V, Pignon JP, Conroy T, Bouche O, Lebreton G, et al. Perioperative chemotherapy compared with surgery alone for resectable gastroesophageal adenocarcinoma: an FNCLCC and FFCD multicenter phase III trial. J Clin Oncol. 2011;29(13):1715-21.
121. Leong T, Smithers BM, Haustermans K, Michael M, Gebski V, Miller D, et al. TOPGEAR: A Randomized, Phase III Trial of Perioperative ECF Chemotherapy with or Without Preoperative Chemoradiation for Resectable Gastric Cancer: Interim Results from an International, Intergroup Trial of the AGITG, TROG, EORTC and CCTG. Ann Surg Oncol. 2017;24(8):2252-8.
122. Cats A, Jansen EPM, van Grieken NCT, Sikorska K, Lind P, Nordsmark M, et al. Chemotherapy versus chemoradiotherapy after surgery and preoperative chemotherapy for resectable gastric cancer (CRITICS): an international, open-label, randomised phase 3 trial. Lancet Oncol. 2018;19(5):616-28.
123. de Steur WO, van Amelsfoort RM, Hartgrink HH, Putter H, Meershoek-Klein Kranenbarg E, van Grieken NCT, et al. Adjuvant chemotherapy is superior to chemoradiation after D2 surgery for gastric cancer in the per-protocol analysis of the randomized CRITICS trial. Ann Oncol. 2021;32(3):360-7.
124. Bang YJ, Kim YW, Yang HK, Chung HC, Park YK, Lee KH, et al. Adjuvant capecitabine and oxaliplatin for gastric cancer after D2 gastrectomy (CLASSIC): a phase 3 open-label, randomised controlled trial. Lancet. 2012;379(9813):315-21.
125. Sakuramoto S, Sasako M, Yamaguchi T, Kinoshita T, Fujii M, Nashimoto A, et al. Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. N Engl J Med. 2007;357(18):1810-20.
126. Yoshida K, Kodera Y, Kochi M, Ichikawa W, Kakeji Y, Sano T, et al. Addition of Docetaxel to Oral Fluoropyrimidine Improves Efficacy in Patients With Stage III Gastric Cancer: Interim Analysis of JACCRO GC-07, a Randomized Controlled Trial. J Clin Oncol. 2019;37(15):1296-304.
127. Paoletti X, Oba K, Burzykowski T, Michiels S, Ohashi Y, Pignon JP, et al. Benefit of adjuvant chemotherapy for resectable gastric cancer: a meta-analysis. Jama. 2010;303(17):1729-37.
128. van Hagen P, Hulshof MC, van Lanschot JJ, Steyerberg EW, van Berge Henegouwen MI, Wijnhoven BP, et al. Preoperative chemoradiotherapy for esophageal or junctional cancer. N Engl J Med. 2012;366(22):2074-84.
129. Shapiro J, van Lanschot JJB, Hulshof M, van Hagen P, van Berge Henegouwen MI, Wijnhoven BPL, et al. Neoadjuvant chemoradiotherapy plus surgery versus surgery alone for oesophageal or junctional cancer (CROSS): long-term results of a randomised controlled trial. Lancet Oncol. 2015;16(9):1090-8.
130. Reynolds JV, Preston SR, O'Neill B, Lowery MA, Baeksgaard L, Crosby T, et al. Neo-AEGIS (Neoadjuvant trial in Adenocarcinoma of the Esophagus and Esophago-Gastric Junction International Study): Preliminary results of phase III RCT of CROSS versus perioperative chemotherapy (Modified MAGIC or FLOT protocol). (NCT01726452). Journal of Clinical Oncology. 2021;39(15_suppl):4004-.
131. Reynolds JV, Preston SR, O'Neill B, Baeksgaard L, Griffin SM, Mariette C, et al. ICORG 10-14: NEOadjuvant trial in Adenocarcinoma of the oEsophagus and

oesophagoGastric junction International Study (Neo-AEGIS). BMC Cancer. 2017;17(1):401.

132. Hoeppner J, Lordick F, Brunner T, Glatz T, Bronsert P, Rothling N, et al. ESOPEC: prospective randomized controlled multicenter phase III trial comparing perioperative chemotherapy (FLOT protocol) to neoadjuvant chemoradiation (CROSS protocol) in patients with adenocarcinoma of the esophagus (NCT02509286). BMC Cancer. 2016;16:503.
133. van den Ende T, Ter Veer E, Mali RMA, van Berge Henegouwen MI, Hulshof M, van Oijen MGH, et al. Prognostic and Predictive Factors for the Curative Treatment of Esophageal and Gastric Cancer in Randomized Controlled Trials: A Systematic Review and Meta-Analysis. Cancers (Basel). 2019;11(4).
134. Camargo MC, Kim WH, Chiaravalli AM, Kim KM, Corvalan AH, Matsuo K, et al. Improved survival of gastric cancer with tumour Epstein-Barr virus positivity: an international pooled analysis. Gut. 2014;63(2):236-43.
135. Derks S, Liao X, Chiaravalli AM, Xu X, Camargo MC, Solcia E, et al. Abundant PD-L1 expression in Epstein-Barr Virus-infected gastric cancers. Oncotarget. 2016;7(22):32925-32.
136. Kim ST, Cristescu R, Bass AJ, Kim KM, Odegaard JI, Kim K, et al. Comprehensive molecular characterization of clinical responses to PD-1 inhibition in metastatic gastric cancer. Nat Med. 2018;24(9):1449-58.
137. Smyth EC, Wotherspoon A, Peckitt C, Gonzalez D, Hulkki-Wilson S, Eltahir Z, et al. Mismatch Repair Deficiency, Microsatellite Instability, and Survival: An Exploratory Analysis of the Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC) Trial. JAMA Oncol. 2017;3(9):1197-203.
138. Choi YY, Kim H, Shin SJ, Kim HY, Lee J, Yang HK, et al. Microsatellite Instability and Programmed Cell Death-Ligand 1 Expression in Stage II/III Gastric Cancer: Post Hoc Analysis of the CLASSIC Randomized Controlled study. Ann Surg. 2019;270(2):309-16.
139. Tsai CY, Lin TA, Huang SC, Hsu JT, Yeh CN, Chen TC, et al. Is Adjuvant Chemotherapy Necessary for Patients with Deficient Mismatch Repair Gastric Cancer?-Autophagy Inhibition Matches the Mismatched. Oncologist. 2020;25(7):e1021-e30.
140. Kim DG, An JY, Kim H, Shin SJ, Choi S, Seo WJ, et al. Clinical Implications of Microsatellite Instability in Early Gastric Cancer. J Gastric Cancer. 2019;19(4):427-37.
141. Pietrantonio F, Miceli R, Raimondi A, Kim YW, Kang WK, Langley RE, et al. Individual Patient Data Meta-Analysis of the Value of Microsatellite Instability As a Biomarker in Gastric Cancer. J Clin Oncol. 2019;37(35):3392-400.
142. Dhakras P, Uboha N, Horner V, Reinig E, Matkowskyj KA. Gastrointestinal cancers: current biomarkers in esophageal and gastric adenocarcinoma. Transl Gastroenterol Hepatol. 2020;5:55.
143. Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer

(ToGA): a phase 3, open-label, randomised controlled trial. Lancet. 2010;376(9742):687-97.

144. Twomey JD, Zhang B. Cancer Immunotherapy Update: FDA-Approved Checkpoint Inhibitors and Companion Diagnostics. AAPS J. 2021;23(2):39.

145. Jiang Y, Xie J, Huang W, Chen H, Xi S, Han Z, et al. Tumor Immune Microenvironment and Chemosensitivity Signature for Predicting Response to Chemotherapy in Gastric Cancer. Cancer Immunol Res. 2019;7(12):2065-73.

146. Wagner AD, Grabsch HI, Mauer M, Marreaud S, Caballero C, Thuss-Patience P, et al. EORTC-1203-GITCG - the "INNOVATION"-trial: Effect of chemotherapy alone versus chemotherapy plus trastuzumab, versus chemotherapy plus trastuzumab plus pertuzumab, in the perioperative treatment of HER2 positive, gastric and gastroesophageal junction adenocarcinoma on pathologic response rate: a randomized phase II-intergroup trial of the EORTC-Gastrointestinal Tract Cancer Group, Korean Cancer Study Group and Dutch Upper GI-Cancer group. BMC Cancer. 2019;19(1):494.

147. Hofheinz RD, Haag GM, Ettrich TJ, Borchert K, Kretzschmar A, Teschendorf C, et al. Perioperative trastuzumab and pertuzumab in combination with FLOT versus FLOT alone for HER2-positive resectable esophagogastric adenocarcinoma: Final results of the PETRARCA multicenter randomized phase II trial of the AIO. Journal of Clinical Oncology. 2020;38(15_suppl):4502-.

148. Shitara K, Bang YJ, Iwasa S, Sugimoto N, Ryu MH, Sakai D, et al. Trastuzumab Deruxtecan in Previously Treated HER2-Positive Gastric Cancer. N Engl J Med. 2020;382(25):2419-30.

149. FDA. FDA approves fam-trastuzumab deruxtecan-nxki for HER2-positive gastric adenocarcinomas. FDA website. Published January 15 AJ, 2021. https://www.fda.gov/drugs/drug-approvals-and-databases/fda-approves-fam-trastuzumab-deruxtecan-nxki-her2-positive-gastric-adenocarcinomas. [

150. Wilke H, Muro K, Van Cutsem E, Oh SC, Bodoky G, Shimada Y, et al. Ramucirumab plus paclitaxel versus placebo plus paclitaxel in patients with previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (RAINBOW): a double-blind, randomised phase 3 trial. Lancet Oncol. 2014;15(11):1224-35.

151. Fuchs CS, Tomasek J, Yong CJ, Dumitru F, Passalacqua R, Goswami C, et al. Ramucirumab monotherapy for previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (REGARD): an international, randomised, multicentre, placebo-controlled, phase 3 trial. Lancet. 2014;383(9911):31-9.

152. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010;363(8):711-23.

153. Zayac A, Almhanna K. Esophageal, gastric cancer and immunotherapy: small steps in the right direction? Transl Gastroenterol Hepatol. 2020;5:9.

154. Smyth EC, Nilsson M, Grabsch HI, van Grieken NC, Lordick F. Gastric cancer. Lancet. 2020;396(10251):635-48.

155. Janjigian YY, Shitara K, Moehler M, Garrido M, Salman P, Shen L, et al. First-line nivolumab plus chemotherapy versus chemotherapy alone for advanced gastric, gastro-oesophageal junction, and oesophageal adenocarcinoma (CheckMate 649): a randomised, open-label, phase 3 trial. Lancet. 2021;398(10294):27-40.
156. Sun JM, Shen L, Shah MA, Enzinger P, Adenis A, Doi T, et al. Pembrolizumab plus chemotherapy versus chemotherapy alone for first-line treatment of advanced oesophageal cancer (KEYNOTE-590): a randomised, placebo-controlled, phase 3 study. Lancet. 2021;398(10302):759-71.
157. Kelly RJ, Ajani JA, Kuzdzal J, Zander T, Van Cutsem E, Piessen G, et al. Adjuvant Nivolumab in Resected Esophageal or Gastroesophageal Junction Cancer. N Engl J Med. 2021;384(13):1191-203.
158. Smyth E, Knodler M, Giraut A, Mauer M, Nilsson M, Van Grieken N, et al. VESTIGE: Adjuvant Immunotherapy in Patients With Resected Esophageal, Gastroesophageal Junction and Gastric Cancer Following Preoperative Chemotherapy With High Risk for Recurrence (N+ and/or R1): An Open Label Randomized Controlled Phase-2-Study. Front Oncol. 2019;9:1320.
159. Chung HC, Bang YJ, C SF, Qin SK, Satoh T, Shitara K, et al. First-line pembrolizumab/placebo plus trastuzumab and chemotherapy in HER2-positive advanced gastric cancer: KEYNOTE-811. Future Oncol. 2021;17(5):491-501.
160. Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med. 1998;4(7):844-7.
161. Nocito A, Kononen J, Kallioniemi OP, Sauter G. Tissue microarrays (TMAs) for high-throughput molecular pathology research. Int J Cancer. 2001;94(1):1-5.
162. Coons AH, Kaplan MH. Localization of antigen in tissue cells; improvements in a method for the detection of antigen by means of fluorescent antibody. J Exp Med. 1950;91(1):1-13.
163. Nakane PK, Pierce GB, Jr. Enzyme-labeled antibodies: preparation and application for the localization of antigens. J Histochem Cytochem. 1966;14(12):929-31.
164. Matos LL, Trufelli DC, de Matos MG, da Silva Pinhal MA. Immunohistochemistry as an important tool in biomarkers detection and clinical practice. Biomark Insights. 2010;5:9-20.
165. Berntsson J, Eberhard J, Nodin B, Leandersson K, Larsson AH, Jirstrom K. Expression of programmed cell death protein 1 (PD-1) and its ligand PD-L1 in colorectal cancer: Relationship with sidedness and prognosis. Oncoimmunology. 2018;7(8):e1465165.
166. Wang L, Zhang Q, Ni S, Tan C, Cai X, Huang D, et al. Programmed death-ligand 1 expression in gastric cancer: correlation with mismatch repair deficiency and HER2-negative status. Cancer Med. 2018.
167. Tretiakova M, Fulton R, Kocherginsky M, Long T, Ussakli C, Antic T, et al. Concordance study of PD-L1 expression in primary and metastatic bladder carcinomas: comparison of four commonly used antibodies and RNA expression. Mod Pathol. 2018;31(4):623-32.

168. Schoemig-Markiefka B, Eschbach J, Scheel AH, Pamuk A, Rueschoff J, Zander T, et al. Optimized PD-L1 scoring of gastric cancer. Gastric Cancer. 2021;24(5):1115-22.
169. Cross SS. Observer accuracy in estimating proportions in images: implications for the semiquantitative assessment of staining reactions and a proposal for a new system. J Clin Pathol. 2001;54(5):385-90.
170. Gavrielides MA, Gallas BD, Lenz P, Badano A, Hewitt SM. Observer variability in the interpretation of HER2/neu immunohistochemical expression with unaided and computer-aided digital microscopy. Arch Pathol Lab Med. 2011;135(2):233-42.
171. Riber-Hansen R, Vainer B, Steiniche T. Digital image analysis: a review of reproducibility, stability and basic requirements for optimal results. APMIS. 2012;120(4):276-89.
172. Nam SJ, Go H, Paik JH, Kim TM, Heo DS, Kim CW, et al. An increase of M2 macrophages predicts poor prognosis in patients with diffuse large B-cell lymphoma treated with rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone. Leuk Lymphoma. 2014;55(11):2466-76.
173. Montaldo E, Del Zotto G, Della Chiesa M, Mingari MC, Moretta A, De Maria A, et al. Human NK cell receptors/markers: a tool to analyze NK cell development, subsets and function. Cytometry A. 2013;83(8):702-13.
174. Langer R, Becker K. Tumor regression grading of gastrointestinal cancers after neoadjuvant therapy. Virchows Arch. 2018;472(2):175-86.
175. Chirieac LR, Swisher SG, Ajani JA, Komaki RR, Correa AM, Morris JS, et al. Posttherapy pathologic stage predicts survival in patients with esophageal carcinoma receiving preoperative chemoradiation. Cancer. 2005;103(7):1347-55.
176. Tomczak K, Czerwinska P, Wiznerowicz M. The Cancer Genome Atlas (TCGA): an immeasurable source of knowledge. Contemp Oncol (Pozn). 2015;19(1A):A68-77.
177. Concato J, Peduzzi P, Holford TR, Feinstein AR. Importance of events per independent variable in proportional hazards analysis. I. Background, goals, and general strategy. J Clin Epidemiol. 1995;48(12):1495-501.
178. Peduzzi P, Concato J, Feinstein AR, Holford TR. Importance of events per independent variable in proportional hazards regression analysis. II. Accuracy and precision of regression estimates. J Clin Epidemiol. 1995;48(12):1503-10.
179. Vittinghoff E, McCulloch CE. Relaxing the rule of ten events per variable in logistic and Cox regression. Am J Epidemiol. 2007;165(6):710-8.
180. Fristedt R, Borg D, Hedner C, Berntsson J, Nodin B, Eberhard J, et al. Prognostic impact of tumour-associated B cells and plasma cells in oesophageal and gastric adenocarcinoma. J Gastrointest Oncol. 2016;7(6):848-59.
181. Cabrita R, Lauss M, Sanna A, Donia M, Skaarup Larsen M, Mitra S, et al. Tertiary lymphoid structures improve immunotherapy and survival in melanoma. Nature. 2020;577(7791):561-5.
182. Sautes-Fridman C, Petitprez F, Calderaro J, Fridman WH. Tertiary lymphoid structures in the era of cancer immunotherapy. Nat Rev Cancer. 2019;19(6):307-25.

183. Yamakoshi Y, Tanaka H, Sakimura C, Deguchi S, Mori T, Tamura T, et al. Immunological potential of tertiary lymphoid structures surrounding the primary tumor in gastric cancer. Int J Oncol. 2020;57(1):171-82.
184. Hiraoka N, Ino Y, Yamazaki-Itoh R, Kanai Y, Kosuge T, Shimada K. Intratumoral tertiary lymphoid organ is a favourable prognosticator in patients with pancreatic cancer. Br J Cancer. 2015;112(11):1782-90.
185. DeNardo DG, Ruffell B. Macrophages as regulators of tumour immunity and immunotherapy. Nat Rev Immunol. 2019;19(6):369-82.
186. Harada K, Dong X, Estrella JS, Correa AM, Xu Y, Hofstetter WL, et al. Tumor-associated macrophage infiltration is highly associated with PD-L1 expression in gastric adenocarcinoma. Gastric Cancer. 2018;21(1):31-40.
187. Leduc C, Adam J, Louvet E, Sourisseau T, Dorvault N, Bernard M, et al. TPF induction chemotherapy increases PD-L1 expression in tumour cells and immune cells in head and neck squamous cell carcinoma. ESMO Open. 2018;3(1):e000257.
188. Mesnage SJL, Auguste A, Genestie C, Dunant A, Pain E, Drusch F, et al. Neoadjuvant chemotherapy (NACT) increases immune infiltration and programmed death-ligand 1 (PD-L1) expression in epithelial ovarian cancer (EOC). Ann Oncol. 2017;28(3):651-7.
189. Lo CS, Sanii S, Kroeger DR, Milne K, Talhouk A, Chiu DS, et al. Neoadjuvant Chemotherapy of Ovarian Cancer Results in Three Patterns of Tumor-Infiltrating Lymphocyte Response with Distinct Implications for Immunotherapy. Clin Cancer Res. 2017;23(4):925-34.
190. Fukuoka E, Yamashita K, Tanaka T, Sawada R, Sugita Y, Arimoto A, et al. Neoadjuvant Chemotherapy Increases PD-L1 Expression and CD8(+) Tumor-infiltrating Lymphocytes in Esophageal Squamous Cell Carcinoma. Anticancer Res. 2019;39(8):4539-48.
191. Yu Y, Ma X, Zhang Y, Zhang Y, Ying J, Zhang W, et al. Changes in Expression of Multiple Checkpoint Molecules and Infiltration of Tumor Immune Cells after Neoadjuvant Chemotherapy in Gastric Cancer. J Cancer. 2019;10(12):2754-63.
192. Garcia-Martinez E, Gil GL, Benito AC, Gonzalez-Billalabeitia E, Conesa MA, Garcia Garcia T, et al. Tumor-infiltrating immune cell profiles and their change after neoadjuvant chemotherapy predict response and prognosis of breast cancer. Breast Cancer Res. 2014;16(6):488.
193. Wei Q, Xu Q, Yuan X, Li JJ, Chen L, Luo C, et al. Immunological impact of chemotherapy on the tumor microenvironment in gastric cancer. J Surg Oncol. 2021;123(8):1708-15.
194. Ladoire S, Arnould L, Apetoh L, Coudert B, Martin F, Chauffert B, et al. Pathologic complete response to neoadjuvant chemotherapy of breast carcinoma is associated with the disappearance of tumor-infiltrating foxp3+ regulatory T cells. Clin Cancer Res. 2008;14(8):2413-20.
195. Pelekanou V, Carvajal-Hausdorf DE, Altan M, Wasserman B, Carvajal-Hausdorf C, Wimberly H, et al. Effect of neoadjuvant chemotherapy on tumor-infiltrating lymphocytes and PD-L1 expression in breast cancer and its clinical significance. Breast Cancer Res. 2017;19(1):91.

About the author

I am a medical oncologist at Halland Hospital and received part of my training at Skåne University Hospital. My clinical area of expertise is gastrointestinal and breast cancer. The focus of my research is to explore the tumor immune microenvironment in gastric and esophageal cancers for improvement of prognostication and response prediction.

Printed by Media-Tryck, Lund 2021 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

FACULTY OF MEDICINE

Department of Clinical Sciences, Lund

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2021:153
ISBN 978-91-8021-160-4
ISSN 1652-8220