Prognostic biomarkers in upper gastrointestinal adenocarcinoma

HEDNER, CHARLOTTA

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Prognostic biomarkers in upper gastrointestinal adenocarcinoma

Charlotta Hedner

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Tampere University Hospital, Finland

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Abstract

Estimates indicate that the global cancer burden may be ever-increasing. Gastric cancer is one of the major cancer forms. Despite its declining incidence, the high mortality of gastric cancer makes it the third most common cause of cancer-related death. Esophageal cancer is less common, but the incidence is increasing in parts of the world. Although some progress has been made in the treatment of these cancer forms, survival rates between 10 and 27 percent confer a dismal prognosis for the afflicted patients.

The main aim of this thesis is to study the prognostic and predictive value of selected biomarkers in upper gastrointestinal cancer in order to identify novel, clinically relevant subgroups of the disease.

Tissue microarrays were created with primary tumours from two consecutive cohorts, one consisting of patients surgically treated for adenocarcinoma in the esophagus or stomach without prior neoadjuvant treatment and the other consisting of patients surgically treated for adenocarcinoma in the esophagus or stomach after neoadjuvant therapy, both in the University hospitals of Lund and Malmö. In addition, a subset of paired normal tissue, pre-treatment biopsies, intestinal metaplasia and lymph node, as well as distant metastases, was sampled. Further, by means of Western blot analysis, siRNA-mediated knockdown, qPCR and immunocyto- and immunohistochemistry, the specificities of the Special AT-rich Sequence-binding Protein (SATB) 1, SATB2 and Human Epidermal Growth Factor Receptor (HER) 3 antibodies used were confirmed.

Immunohistochemichal expression of SATB1, a global genome organiser that has been demonstrated to promote aggressive tumour behaviour in several types of cancer, was shown to be an independent adverse prognostic biomarker in patients with radically resected adenocarcinomas of the esophagus and stomach. The distribution, interrelationship and prognostic significance of protein expression and gene amplification of the treatment target HER2 was examined in the tumours from the first cohort. Expression of HER2 in primary tumours had no prognostic impact, whereas conversion of expression between primary tumour and lymph node metastasis was an independent adverse prognostic factor. The expression of EGFR (Epidermal Growth Factor Receptor 1) and HER3 in tissue from the first cohort was also examined. EGFR was independently associated with a shorter overall survival. High HER3 expression was associated with a longer overall survival, although not independently. The expression, interrelationship and prognostic significance of EGFR, HER2 and HER3 was also examined in the tumours from the second cohort. No associations between EGFR or HER2 expression and survival were seen. A non-independent association between post-treatment HER3 expression and longer overall survival was seen. A change in expression of the examined proteins between pre-treatment biopsies and post-treatment resection specimens was seen in 5, 6 and 20% of the cases, respectively.

In conclusion, our results provide further evidence that SATB1 expression is associated with poor prognosis. Our studies also shed light on new aspects of HER expression, associations with prognosis and changes in expression during the growth, spread and treatment of tumours, which could affect diagnostic and treatment strategies.

Key words: Esophgaeal adenocarcinoma, gastric adenocarcinoma, SATB1, EGFR, HER2, HER3

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Prognostic biomarkers in upper gastrointestinal adenocarcinoma

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When I published the results of my experiments on the development of double-fertilized sea-urchin eggs in 1902, I added the suggestion that malignant tumours might be the result of a certain abnormal condition of the chromosomes, which may arise from multipolar mitosis… So I have carried on for a long time the kind of experiments I suggested, which are so far without success, but my conviction remains unshaken. – Theodor Boveri, pathologist, 1914

Cell-fie

Contents

List of papers

Papers included in the thesis. The papers are referred to in the text by their respective Roman numerals:

I.   Hedner C, Gaber A, Korkocic D et al. SATB1 is an independent prognostic factor in radically resected upper gastrointestinal tract adenocarcinoma. Virchows Archiv 465:649-59 (2014).

II.   Hedner C, Tran L, Borg D, Nodin B, Jirstrom K, Eberhard J. Discordant human epidermal growth factor receptor 2 overexpression in primary and metastatic upper gastrointestinal adenocarcinoma signifies poor prognosis. Histopathology 68: 230-40 (2015).

III.   Hedner C, Borg D, Nodin B, Karnevi E, Jirstrom K, Eberhard J. Expression and prognostic significance of human epidermal growth factor receptors 1 and 3 in gastric and esophageal adenocarcinoma. PLoS One 11:e0148101 (2016).

IV.   Hedner C, Borg D, Nodin B, Karnevi E, Jirstrom K, Eberhard J. Expression and prognostic significance of human epidermal growth factor receptors 1, 2 and 3 in esopgaheal and gastric adenocarcinomas pre- and post neoadjuvant treatment. Submitted.

Papers not included in the thesis

- Nodin B, Hedner C, Uhlen M, Jirstrom K: Expression of the global regulator SATB1 is an independent factor of poor prognosis in high grade epithelial ovarian cancer. Journal of ovarian research, 5:24 (2012).

- Hedner C, Sundgren PC, Kelly AM: Associations between presence of relevant information in referrals to radiology and prevalence rates in patients with suspected pulmonary embolism. Academic radiology, 20:1115-1121 (2013).

- Jonsson L, Hedner C, Gaber A et al. High expression of RNA-binding motif protein 3 in esophageal and gastric adenocarcinoma correlates with intestinal metaplasia-associated tumours and independently predicts a reduced risk of recurrence and death. Biomarker research 2:11 (2014).

- Fristedt R, Gaber A, Hedner C et al. Expression and prognostic significance of the polymeric immunoglobulin receptor in esophageal and gastric adenocarcinoma. Journal of translational medicine 12:83 (2014).

- Borg D, Hedner C, Gaber A, Nodin B, Fristedt R, Jirstrom K, Eberhard J, Johnsson A. Expression of IFITM1 as a prognostic biomarker in resected gastric and esophageal adenocarcinoma. Biomarker research 14:10 (2016).

- Borg D, Hedner C, Nodin B, Larsson A, Johnsson A, Eberhard J, Jirstrom K. Expression of podocalyxin-like protein is an independent prognostic biomarker in resected esophageal and gastric adenocarcinoma. BMC Clinical Pathology 16:13 (2016)

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Abbreviations

A	Adenine nucleotide
AJCC	American Joint Committee on Cancer
AKT	Protein kinase B
ATP	Adenosine triphosphate
BE	Barrett's esophagus
BRAF	v-RAF murine sarcoma viral oncogene homologue B1
C	Cytosine nucleotide
CAIRO	Capecitabine, Irinotecan, and Oxaliplatin in Advanced Colorectal Cancer
CI	Confidence interval
CISH	Chromogenic in situ hybridisation
CT	Computed tomography
D1	Limited gastric lymphadenectomy including lymph node stations 1-6
D2	Extended gastric lymphadenectomy including lymph node stations 1-11
DNA	Deoxyribonucleic acid
cDNA	Complimentary DNA
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FISH	Fluorescence in situ hybridisation
GTP	Guanosine triphosphate
HER	Human epidermal growth factor receptor
HP	Helicobacter Pylori
HR	Hazard ratio
IHC	Immunohistochemistry
IM	Intestinal metaplasia
ISH	In situ hybridisation
Ki67	Antigen KI67
KM	Kaplan Meier
KRAS	Kirsten rat sarcoma viral oncogene homologue
MAB	Monoclonal antibody
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
MLH1	mutL homologue 1
MSI	Microsatellite instability
M-stage	Distant metastasis
N-stage	Lymph node involvement
OS	Overall survival

P13K	Phosphatidylinositol-4,5-bisphosphonate 3-kinase
p53	Tumour protein 53
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
rtPCR	Reverse transcription PCR
PET	Positron emission tomography
PSA	Prostate specific antigen
RECIST	Response evaluation criteria in solid tumours
RFS	Recurrence free survival
RNA	Ribonucleic acid
mRNA	messenger ribonucleic acid
siRNA	small interfering ribonucleic acid
RTOG	Radiation Therapy Oncology Group
SATB	Special AT-rich sequence binding protein
SISH	Silver in situ hybridisation
SNPs	Single-nucleotide polymorphisms
T	Thymine nucelotide
TKI	Tyrosine kinase inhibitor
TMA	Tissue microarray
TNM	Tumour node metastasis
cTNM	clinical TNM stage
pTNM	pathological TNM stage
ypTNM	pathological TNM stage of the surgical specimen after neoadjuvant treatment
TRG	Tumour regression grading
T-stage	Tumour stage
TTR	Time to recurrence
UICC	Union for International Cancer Control
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WHO	World Health Organization

Background

Cancer

A recent World Health Organization (WHO) report estimates the global cancer burden to be ever-increasing[1]. However, if predisposing lifestyle factors (such as smoking, alcohol abuse and obesity) as well as infectious agents (such as Helicobacter pylori (HP) and hepatitis) could be eliminated and screening programs for precancerous lesions implemented, it is estimated that more than half of all cancer cases could be prevented[2]. Cancer is a group of diseases characterised by an uncontrolled growth and spread of abnormal cells, and all cancers are genetic in the sense that they involve DNA alterations leading to the malfunction of genes that control cell growth, division and death[2]. This malfunction of genes can be caused by both external factors (such as tobacco, chemicals, radiation and infectious agents) and internal factors (such as inherited or acquired genetic alterations or immune conditions)[2]. Most of the genetic abnormalities that affect cancer risk are not hereditary, and most cancers develop through multiple changes resulting from a combination of hereditary and environmental factors[2]. It is estimated that around 15% of all cancer, more in developing than in developed countries, is attributable to infections[2].

The genes involved in genetic changes can be classified into three types: oncogenes, tumour suppressor genes and deoxyribonucleic acid (DNA) repair genes[3]. Under normal conditions oncogenes can stimulate appropriate cell growth, but mutation or overexpression results in a gain of function, which causes unregulated cell growth[3]. Tumour suppressor genes normally inhibit progression through the cell cycle or promote apoptosis, and loss of their function results in loss of normal inhibitory control. DNA repair genes are, when malfunctioning, unable to repair errors in the DNA, leading to accumulation of mutations in oncogenes and tumour suppressor genes[3].

Cancer survival rates are affected by several factors, the most important of which are type of cancer, stage at diagnosis and whether treatment is available. For cancers that are affected by screening and/or treatment, there are large survival differences between developed and developing countries[2].

Tumour stageing

Tumour stage describes the extent, or spread, of the disease at the time of diagnosis. The most widely used staging system is the tumour node metastasis (TNM) system[4], which assesses tumours in three ways: size and/or extent of the primary tumour (T), absence or presence of regional lymph node metastases (N) and absence or presence of distant metastases (M)[2]. The system varies between tumour types, with only tumour size in millimeters determining T-stage for some cancer types and depth of invasion through anatomical layers, or a combination of these, for other types. After determination of TNM stage, a disease stage I (early), II, III or IV (advanced) is assigned[2]. Traditionally, treatment decisions have been based primarily on stage, however, as the molecular properties of cancer have become better understood, biological markers and genetic features are now also being taken into consideration in treatment decisions[2]. Before surgical resection, a clinical TNM (cTNM) stage is assigned based on available clinical information, radiology and diagnostic biopsies. After surgical resection, a pathologic TNM (pTNM) stage is assigned[4], which provides the basis for prognostication and treatment decision. If the patient has received neoadjuvant treatment, the surgical specimen will be classified according to ypTNM instead[4]. The lack of a pTNM stage in such cases raises the question of whether cTNM or ypTNM is the better prognostic tool, something that has not yet been firmly established[5].

Tumour regression

As a further prognostic tool, radiological as well as pathological evaluation systems for assessment of treatment response have been introduced[6]. The pathological tumour regression grading (TRG) systems aim to categorise the amount of regressive changes after cytotoxic treatment in order to demonstrate potential prognostic information, and they refer mostly to the amount of therapy-induced fibrosis in relation to residual tumour or the estimated percentage of residual tumour in relation to the previous tumour site[7]. There are several TRG systems in use for different tumour types, for example for esophageal cancer, gastric cancer and rectal cancer, and several studies indicate regression of the primary tumour to be a very significant prognostic factor[5, 8-12]. Although not all studies have found TRG to be an independent prognostic factor[13], others have even suggested that pathological TRG systems could be the strongest prognostic indicator of all[14]. TRG systems based on percentage of residual tumour have generally been more reproducible than TRG systems based on estimation of fibrosis in relation to residual tumour[7]. Figure 1 illustrates TRG according to Chirieac[5].
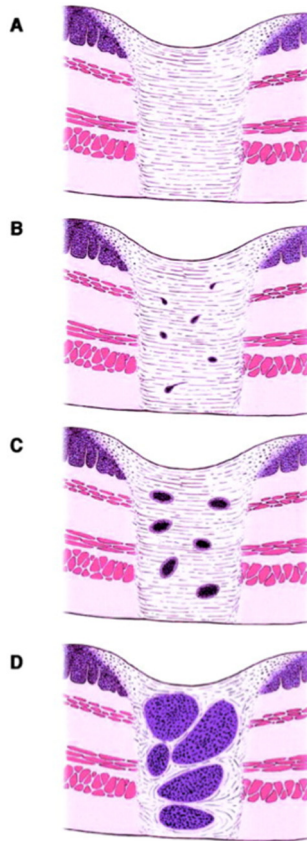
Figure 1
Illustration of the evaluation of tumour regression after neoadjuvant treatment. Schematic cross-sectional view of treated primary tumour, where the extent of residual carcinoma is semiquantitatively assigned to one of four categories: (A) No residual carcinoma, (B) 1-10% residual carcinoma, (C) 11-50% residual carcinoma and (D) Greater than 50% residual carcinoma. Reproduced with permission from Prof Chirieac.

Esophageal and gastric cancer

Incidence and epidemiology

Esophageal cancer, comprising both squamous cell cancer and adenocarcinoma, is the eighth most common cancer type worldwide, but its high mortality rates make it the sixth most common cause of cancer-related death[1]. The incidence rates vary greatly both across sex and world region, with 3-fold higher incidence rates in men than in women and up to 20-fold differences between world regions, with around 80% of the cases occurring in developing regions, particularly in Asia[1].

Globally, squamous cell cancer predominates over adenocarcinoma[1, 2, 15]. However, in many western countries, squamous cell cancer is sharply declining, whereas adenocarcinoma is increasing and has been escalating with a rate higher than that of nearly all other types of cancer[15, 16]. In Sweden, the number of adenocarcinomas in the esophagus now surpasses that of squamous cell carcinomas[15]. Figure 2 illustrates global variations in esophageal cancer incidence.
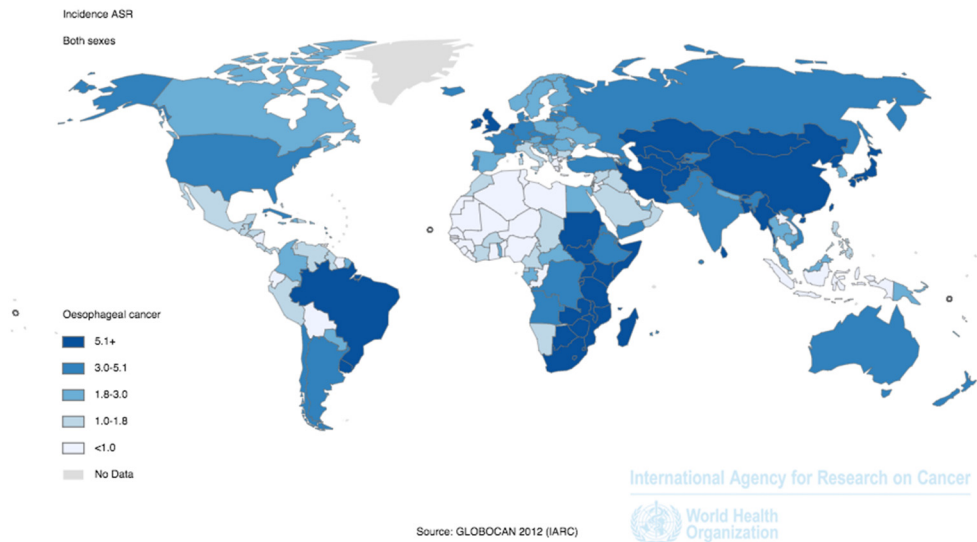
Figure 2. Global variations in esophageal cancer incidence[1]. Reprinted with permission from GLOBOCAN.

The incidence of gastric cancer is declining, but it is still the fifth most common malignancy in the world, with around 950 000 new cases presenting each year, and the third most common cause of cancer-related death[1, 17]. Gastric cancer is to 95% comprised of adenocarcinomas[15]. Similarly to esophageal cancer, incidence rates vary significantly across countries, with the highest rates in Asia and parts of South America, and the lowest rates in North America and parts of Africa. The incidence is about twice as high in males as in females[1]. Figure 3 illustrates global variations in gastric cancer incidence.

Incidence ASR

Both sexes

Stomach cancer
- 11.0+
- 7.4-11.0
- 5.2-7.4
- 3.5-5.2
- <3.5
- No Data

Source: GLOBOCAN 2012 (IARC)

International Agency for Research on Cancer
World Health Organization
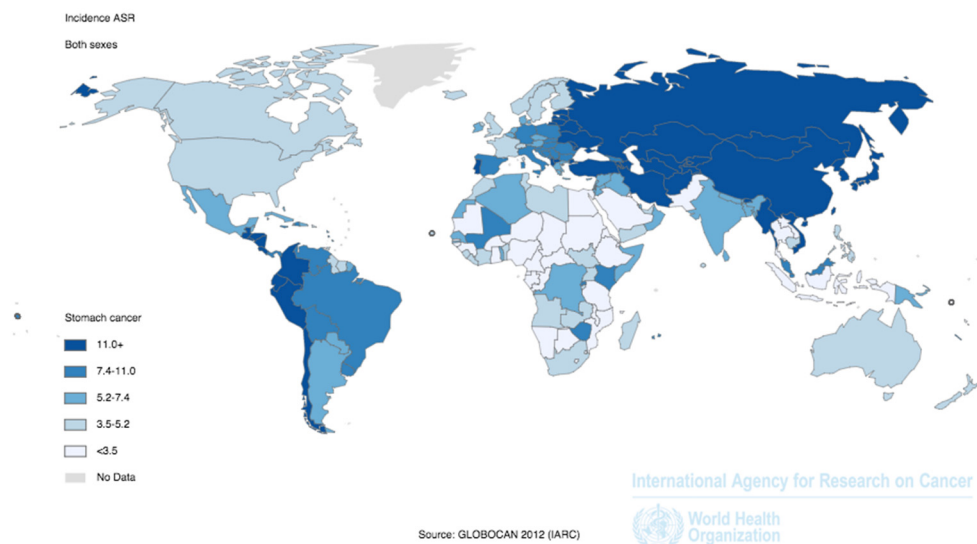
Figure 3.
Global variations in gastric cancer incidence[1]. Reprinted with permission from GLOBOCAN.

Etiology

Gastric HP infection is a risk factor for gastric adenocarcinoma but has been linked to a reduced risk for esophageal adenocarcinoma, and the increasing incidence of esophageal adenocarcinoma has been suggested to be linked to declining rates of HP infection in the Western society[18, 19].

Several studies suggest that the mechanism for this may be that HP infection induces atrophic gastritis, in turn causing loss of gastric acidity, thus protecting against gastroesophageal reflux and reducing the occurence of Barrett's esophagus (BE)[18-20]. BE, which is strongly associated with gastroesophageal reflux, is a metaplastic change of the esophageal epithelium from normal squamous to intestinalised columnar mucosa[21]. Patients with BE have a risk of developing adenocarcinoma via widespread genomic instability, involving both tumour suppressor genes and oncogenes, with an annual rate of neoplastic transformation of approximately 0.5%[21, 22]. However, BE *per se* is asymptomatic. A problem with using reflux symptoms as a marker for increased risk of developing adenocarcinoma is that, since reflux symptoms are so common and adenocarcinoma relatively rare, the absolute risk of developing adenocarcinoma for individuals with reflux symptoms is very low. Of note, up to 40% of those with esophageal adenocarcinoma do not have weekly reflux symptoms[21, 23]. Obesity is also correlated to an increased risk of esophageal adenocarcinoma, where it has been postulated that an increased abdominal pressure contributes to

gastroesophageal reflux[24]. Factors lowering the risk for esophageal adenocarcinoma are diets rich in fruit and vegetables[15].

HP infection, that is a major risk factor for gastric cancer, causes atrophic gastritis followed by gastric intestinal metaplasia and adenocarcinoma, which can be attributed to more than 50% of the gastric adenocarcinoma cases[2]. Other risk factors include cigarette smoking and diets rich in smoked or salted food[25]. In addition to environmental factors, genetic factors also play an important role in the development of esophageal and gastric cancer, both via genetic susceptibility and via acquisition of genetic and epigenetic alterations[26]. Apropos genetic susceptibility, single-nucleotide polymorphisms (SNPs), for example in the vascular endothelial growth factor (VEGF) gene that is involved in the genetic susceptibilty to gastric cancer, are genetic variants that may modulate the effects of environmental factors by regulating biological pathways in response to exposure, thus exerting an effect on population attributable risks[26]. Regarding molecular alterations, although the molecular pathogenesis of gastric cancer is incompletely understood, a number of alterations have been identified, namely gene overexpression (such as human epidermal growth factor receptor 2 (HER2) and histone modifying enzymes), gene silencing, and microsatellite instability (MSI)- associated gene mutations[26]. A small number of patients may have a genetic predisposition syndrome[25]. Familial clustering is observed in approximately 10% of gastric cancers, but hereditary gastric cancer accounts for only 1%-3% of cases, where gastric cancer can be part of inherited cancer syndromes such as familial adenomatous polyposis, Peutz-Jeghers and MSI-related hereditary nonpolyposis colon cancer/Lynch syndrome[16, 25, 27, 28].

Signs and symptoms

Common clinical presentation symptoms for esophageal cancer are dysphagia and weight loss[24, 29]. Other symptoms include reflux, chest pain, painful swallowing and anemia[16, 24]. In symptomatic gastric cancer patients, the presenting features commonly include weight loss, dysphagia, vomiting, early satiety, and/or iron-deficiency anaemia[25, 30]. Early stages can include indigestion and heartburn, or they may be asymptomatic[2, 30]. Unfortunately, many cases of esophageal and gastric cancer are diagnosed at an advanced stage, partly because the tumours tend to give very unspecific early symptoms, and partly due to patient's delay on account of failure to recognise the gravity of the symptoms[29].

Diagnostics

The golden standard for diagnosis of esophageal or gastric cancer is from an endoscopic or surgical biopsy[25, 31]. Since therapeutic strategy is based on clinical staging, efforts are made to assess the pre-theraputic tumour stage by clinical examination, blood tests, computed tomography (CT) and, in some cases, endoscopic ultrasound and/or positron emission tomography (PET)-CT and laparoscopy[25, 31]. Nevertheless, clinical staging is difficult and the accuracy of clinical N-staging does not exceed 80%[31]. The stage is to be determined according to the American Joint Committee in Cancer (AJCC) TNM system (7th ed)[25, 31].

Tumour stage (T-stage)

A schematic illustration of T-stages of esophageal and gastric cancer according to the AJCC/Union for International Cancer Control (UICC) 7th Edition is provided in Figure 4[32].
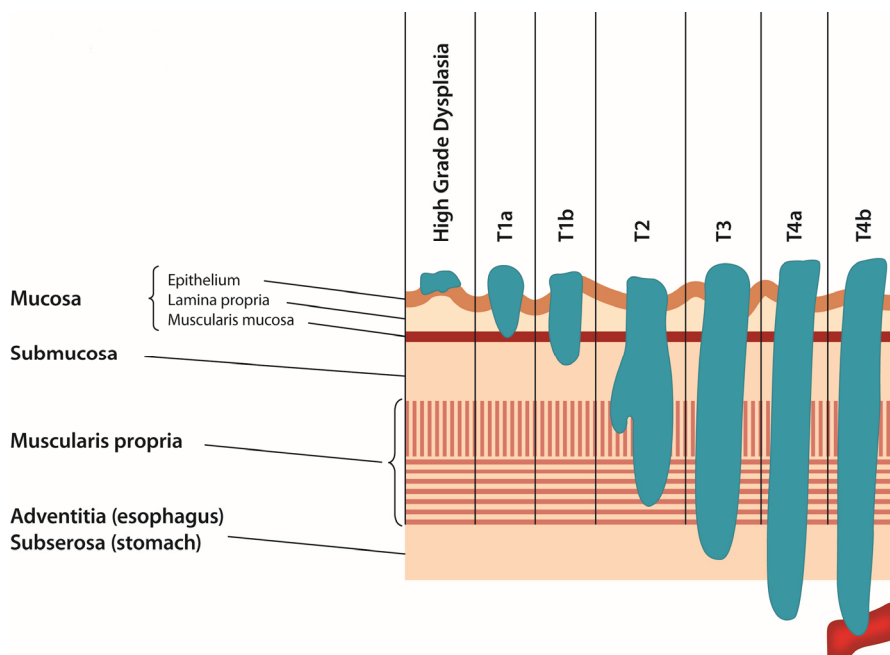

Figure 4
T-stages of esophageal and gastric cancer. Reproduced wiith permission from C Runehammar.

Lymph node involvment (N-stage) and distant metastasis (M-stage)

According to the AJCC/UICC 7th Edition, N-staging for esophageal as well as gastric cancer is performed as follows: N0 – No regional lymph node metastasis, N1 – Metastasis in 1-2 regional lymph nodes, N2 – metastasis in 3-6 regional lymph nodes and N3 – metastasis in 7 or more regional lymph nodes. M0 is classified as the absence of distant metastasis and M1 as the presence of distant metastasis[32]. Of note, the lymph node stations classified as regional differ somewhat between esophageal and gastric cancer, thus affecting N- and M-staging[32].

Other relevant clinicopathological factors

Apart from TNM stage, some additional factors that may affect prognosis are included in the pathology report[15].

Several classification systems of gastric adenocarcinoma have been proposed, most of which are primarily based on the microscopic appearance of the tumour, the most widely used being the Laurén and WHO classifications[22]. The Laurén classification is generally applied on gastric cancer, but can also be used for esophageal cancer[14]. In this thesis, tumour histological growth pattern was classified according to Laurén and denoted as intestinal, diffuse or mixed. The diffuse type generally has a worse prognosis than the intestinal type[33].

Swedish pathology guidelines recommend that pathologic response to treatment be assessed, e.g. according to Becker or Wu[15, 34], whereas other guidelines do not include this recommendation. The degree of pathologic response does however not have any implication for treatment strategy according to current European guidelines[25, 31]. In this thesis, histopathologic response was assessed according to Chirieac in the neoadjuvantly treated surgical resection specimens from Cohort 2.

Differentiation grade, perineural or intravascular tumour growth, and resection margins are additional factors with prognostic impact that are also denoted in the pathology report[15, 35].

Heterogeneity

An issue that can complicate tumour diagnostics is heterogeneity. Tumour heterogeneity entails differences between tumours of the same type in different patients, between cancer cells within a tumour, or between different tumour cell populations within a patient. One important aspect of tumour heterogeneity is whether the sample taken from a patient for analysis is representative of the

driving properties of the tumour. It has also been suggested that heterogeneity *per se* could be an adverse prognostic factor, which is difficult to target[36]. This is exemplified in the results from the Capecitabine, Irinotecan and Oxaliplatin in Advanced Colorectal Cancer (CAIRO) colorectal studies[37], where patients with disseminated colorectal cancer and a heterogeneous partial response to treatment had a worse prognosis than patients with a homogeneous partial response. Response was defined according to the response evaluation criteria in solid tumours (RECIST) criteria, with partial response being defined as at least two lesions within a patient showing a different behaviour, i.e. +10% progress versus -10% response. A shorter recurrence-free survival (RFS) for patients with heterogeneous HER2 amplification than for those with homogeneous HER2 amplification has been seen in a study of breast cancer[38]. However, very few studies have addressed the prognostic significance of intratumoural heterogeneity in gastric cancer[36, 39, 40]. Unfortunately there is no consensus regarding how heterogeneity is to be defined[39], making an equitable picture difficult to ascertain. As an example, Rüschoff *et al.* have defined HER2 heterogeneity in gastric cancer as <30% positive staining of tumour cells[41]. Other studies have used different definitions of HER2 heterogeneity in gastric cancer[39, 42, 43], and the wide range (1–75%) of reported heterogeneity[36, 42] may therefore not be accurate, although it seems fairly well established that heterogeneity is more common in gastric carcinoma than in for example breast cancer[36, 44-46]. Not only can heterogeneity within a primary tumour be seen, but metastatic progression could cause heterogeneity to become more pronounced, causing a discordance between a primary tumour and a metastasis, thus raising the question of which lesion should be sampled in order to identify the most useful treatment[36]. Concordance studies on the expression of different growth factor receptors in primary and metastatic tumours have been performed in several types of cancer, including gastric cancer. However, most of these studies did not investigate whether the discordance had any prognostic impact[46-52]. Yet another aspect of tumour heterogeneity could be a possible role in therapeutic resistance because of a selection of subclones lacking for example HER2 overexpression, leading to failure of HER2-blocking treatment[38].

In this thesis, intratumoural HER2 heterogeneity and its prognostic significance was examined in Cohort 1. Primary-metastatic heterogeneity was examined for epidermal growth factor receptor (EGFR), HER2 and HER3 in Cohort 1 and 2.

Treatment of esophageal and gastric adenocarcinoma

The treatment of esophageal and gastric cancer is a multidisciplinary collaboration of surgeons, oncologists, pathologists and radiologists. All cases should be discussed at multidisciplinary tumour boards according to current European guidelines[25, 31]. The main factors for selecting primary therapy are tumour stage and location, histological type and the medical condition of, as well as considerations from, the patient[31].

Surgery

Surgery is one of the cornerstones for the management of esophageal and gastric cancer. The goal is to remove the primary tumour with tumour free margins, resect its vascular supply and lymphatic drainage, and re-establish continuity of the digestive tract.

Surgery alone or minimally invasive endoscopic mucosal resections is the treatment of choice for early esophageal and gastric cancer[25, 31]. Since the required surgery can be extensive, the surgical procedure itself can be a cause of mortality, with potential complications such as anastomosal leakage and pneumonia[15]. For example, esophageal resections have hospital-bound mortality rates of up to 10%, but the mortality rates of both esophageal and gastric surgical procedures can be reduced by centralisation of surgery to high-volume centers[15, 53, 54]. The introduction of fast-track or enhanced recovery after surgery programs and laparoscopic techniques are additional strategies to further reduce mortality, that have shown promising results[55]. Several different strategies for resection and reconstruction of the esophagus and stomach exist, but in general the experience and preference of the surgeon affects the outcome more than the type of surgery[15]. The extent of nodal dissection accompanying radical gastrectomy (limited lymphadenectomy including lymph node stations 1-6 (D1) versus extended lymphadenectomy including lymph node stations 1-11 (D2)) has been extensively debated[25]. Current European guidelines recommend that D2 dissection should be the standard procedure in specialised, high-volume centers[25].

The patients included in the cohorts of this thesis have undergone surgical resection. The type of surgery was not included as a factor in the multivariable statistical analyses.

Chemotherapy and radiotherapy

The use of chemotherapy originates from the discovery during World War II that mustard gas is a potent suppressor of hematopoiesis[56]. This finding led researchers to try the substance on patients suffering from leukemia (a malignancy characterised by an increased rate of hematopoiesis). In the 1960's the first patient was cured[56]. For some types of cancer, chemotherapy has radically improved prognosis, whereas for others the effect is more modest[56]. Chemotherapeutic agents have different mechanisms of action and can be divided into four main groups: (1) alkylating agents and platinum compounds that affect the DNA directly, (2) antimetabolites and topoisomerase inhibitors (e.g. fluoropyrimidine/ 5-FU and epirubicin) that affect DNA indirectly, (3) alkaloids and taxanes that inhibit cell division by causing microtubule dysfunction and (4) others such as antibiotic-like agents, that are mostly used for hematopoietic malignancies[56]. Most chemotherapy regimens are based on combinations of agents with different mechanisms of action[56].

The discovery of X-rays and radioactivity and the isolation of radium in the 1890's laid the foundation for the development of radiotherapy, and treatment of patients started already in 1899[56]. The radiation used is ionising and aimed directly against the membrane and DNA of the cell, but since cells consist of 80% water, the radiation may often hit a water molecule instead, giving rise to free radicals, which may indirectly damage the DNA[56]. The sensitivity to radiotherapy differs among tumour types and between different normal tissues, but also varies during the cell cycle[56]. To optimise the effect of radiation, it is also important that the cells are not hypoxic and that the frequency of treatment is attuned to the proliferative rate of the tumour[56]. The absorbed dose is indicated in Gray (Gy), where 1 Gy = 1 joule/kg[56].

Some chemotherapeutic agents enhance the effect of radiotherapy, hence, for some tumour types such as esophageal cancer, a combination of radiotherapy and chemotherapy is commonly used[56].

In recent years several trials have demonstrated an improved overall survival for patients with locally advanced esophageal or gastric tumours receiving neoadjuvant or perioperative chemotherapy and/or chemoradiotherapy, and this is now part of current guidelines[31, 57, 58]. The European standard of treatment for gastric cancer is currently a combination of fluoropyrimidine and a platinum-based agent, such as cisplatin or oxaliplatin, with an optional addition of epirubicin[15, 25]. For esophageal adenocarcinoma, a platinum-based agent + fluoropyrimidine or taxane, optionally in combination with radiotherapy (40-50 Gy), is recommended[15, 31].

Palliative treatment modalities in advanced esophageal or gastric cancer include radiotherapy, chemotherapy and dilatation or insertion of a stent for relief of dysphagia[15]. Palliative chemotherapy with fluoropyrimidine and/or platinum-containing regimens have been the mainstay of advanced gastric cancer management for many years and is associated with an improved overall survival compared with best supportive care alone, with median overall survival times of 10-12 months[59, 60]. Palliative radiotherapy can be given in case of pronounced symptoms from the primary tumour such as dysphagia or bleeding or from skeletal metastases.

None of the patients in Cohort 1 of this thesis received neoadjuvant treatment, but a small percentage received adjuvant and/or palliative treatment. All patients in Cohort 2 received neoadjuvant treatment, the majority of them a combination of platinum-based chemotherapy + fluoropyrimidine, or platinum-based chemoradiotherapy.

Treatment predictive markers and targeted therapy

Although the use of chemotherapy and/or radiotherapy has led to a substantial survival benefit when compared to surgery alone for gastric and esophageal cancer patients, the traditional prognostic clinicopathological characteristics described above provide very limited information on which patients will benefit from this kind of treatment[26, 60].

A targeted cancer therapy is a treatment aimed at specific mechanisms in the cancer cell, with the purpose to block growth or spread of the cancer cells[56]. The aim is to provide a more efficient treatment, with fewer side effects than chemotherapy or radiotherapy, that take advantage of the fact that neoplastic cells tend to proliferate at a higher rate than normal cells, thus affecting all proliferating cells[56]. A challenge for successful targeted therapy has proven to be the identification of tumours where the target molecule is the driver of the tumour growth[56]. The two major groups of targeted drugs are monoclonal antibodies (MABs) and tyrosine kinase inhibitors (TKIs)[56].

So far, two targeted drugs have been approved for the treatment of advanced gastric or gastroesophageal cancer[61]. One is a monoclonal antibody against the HER2 receptor (trastuzumab, approved for first line palliative treatment), where treatment prolongs survival for patients with HER2-positive advanced disease, thus being the only treatment with a validated predictive biomarker[62, 63]. The ongoing Radiation Therapy Oncology Group (RTOG) 1010 trial will provide valuable information on whether giving trastuzumab in the neoadjuvant and adjuvant setting to patients with HER2-overexpressing esophageal adenocarcinoma will further prolong survival[64]. The second targeted drug is a

recently approved (Dec 2014 in the EU[65]) anti-vascular endothelial growth factor receptor 2 (VEGFR2) antibody (ramucirumab, approved for second line palliative treatment), which has expanded the gastric cancer armamentarium. However, there are no validated predictive biomarkers to identify which patients may derive benefit from anti-VEGFR targeted therapies[59, 60]. Several other targeted therapies have been tested in different trials, including examination of the potential benefit of adding an anti-EGFR agent, without significantly improved survival[66, 67]. Other investigated treatment options for gastric cancer are blocking estrogen receptors and histamin-receptors, unfortunately without success[15].

Prognosis

Despite recent advances in perioperative and adjuvant oncological treatment, most patients with advanced gastric cancer have a median survival of less than one year[26], and the five-year overall survival is around 25%[2]. The best prognostic parameters are TNM-staging and grading, as well as the complete surgical removal of the neoplastic tissue[26, 31].

Investigative biomarkers

A biological marker, or biomarker, may be defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention"[68] and can be prognostic as well as predictive of therapeutic response[69]. A well-known example is the use of a cancer biomarker is prostate specific antigen (PSA) in prostate cancer diagnostics and monitoring of treatment effect[56]. Unfortunately, very few molecules have been identified that are expressed only in cancerous tissue and not in the corresponding normal tissue[69]. Despite a large number of published biomarker studies, few cancer biomarkers have been introduced into clinical practise[69].

In this thesis, the candidate biomarkers special AT-rich sequence-binding protein (SATB) 1, SATB2, tumour protein 53 (p53), antigen KI67 (Ki67), Kirsen rat sarcoma viral oncogene homologue (KRAS), EGFR, HER2, and HER3 have been examined.

SATB1

When cells change activity or function, gene expression undergoes reprogramming, which requires changes in chromatin architecture[70]. This involves recruitment of chromatin remodelling enzymes and epigenomic modification enzymes[70]. SATB1 is an organiser protein that provides a nuclear architectural platform where hundreds of proteins can anchor, thus facilitating the change in gene expression[70]. SATB1 was first identified as a protein that binds specifically to 100-300 base pair long sequences of DNA that are characterized by clusters of As, Ts and Cs (ATC-sequences). These are genomic regions with ability to unpair DNA bases and bind to different parts of chromatin strands, thereby organising chromatin into loops and regulating gene transcription[70]. SATB1 is expressed in normal thymocytes[70]. In an *in vivo* experiment knockout of SATB1 prevented normal differentiation into T lymphocytes. Expression of SATB1 in tumour tissue has been correlated to aggressive behaviour in several tumour types[70-74] including gastric cancer[75, 76], although some studies have

demonstrated contrasting results[77-79]. One explanation for the differing results may be methodologic differences[80], but as SATB1 is highly homologous to SATB2, the specificity of the antibodies used has previously been questioned[77]. Presently SATB1 is not in clinical use and prior to this work, SATB1 expression had not been examined in esophageal cancer.

SATB2

SATB2, like SATB1, is a DNA-binding protein that is involved in transcription regulation and chromatin remodeling[81]. Knockout models have shown SATB2 to be involved in normal skeletal development, and dysfunction of SATB2 has been shown to cause cognitive defects as well as craniofacial dysmorphism and osteoporosis[81]. It is also expressed in normal colorectal mucosa and in colorectal adenocarcinomas, but more sparsely in other types of normal tissue and cancer[82]. Although being higly homologous, studies have indicated antagonistic qualities of SATB1 and SATB2 in for example colorectal cancer, where reduced levels of SATB2 have been correlated to poor prognosis[70, 83, 84]. At present SATB2 is not in clinical use.

In this thesis, the expression and prognostic significance of SATB1 and SATB2 was examined in Cohort 1.

P53

P53 is a DNA binding protein and transcription factor that controls the expression of a large number of genes[85, 86]. It has been called "the guardian of the genome" because of its ability to block cell proliferation in the presence of DNA damage[85-87]. A mutation in the p53 gene, in spite of being a loss-of-function mutation, can increase protein stability. Therefore, strong immunohistochemistry (IHC) expression indicates the presence of a mutation, although not all mutations can be detected with IHC[3, 88]. P53 is the most commonly mutated gene in all types of cancer[3, 87]. At present, the presence of p53 mutation can be used as an indicator of transition from low-grade to high-grade dysplasia in BE, but it is not used for prognostication in esophageal or gastric cancer[34, 88]. Targeted therapy against p53 is in clinical development, though as yet only in phase I[63, 89].

In this thesis, the expression and prognostic significance of p53 was investigated in Cohort 1.

Ki67

Ki67, first described in 1983, is a nuclear protein that is expressed in all active phases of the cell cycle, but not in resting cells. Therefore, it is now standard to use Ki67 to index cellular proliferation[87, 90, 91]. In several types of cancer there is an association between high proliferation rates and poor prognosis, and therefore Ki67 has been incorporated into clinical protocols as a prognostic marker[92]. However, its role as a prognostic marker in upper gastrointestinal cancer remains to be established[87].

In this thesis, the expression and prognostic significance of Ki67 was investigated in Cohort 1.

EGFR signalling pathway

Proliferation of cells is tightly regulated through cellular signal transduction pathways[26]. Growth factors and their receptors play important roles in the regulation of these pathways[26]. The human epidermal growth factor receptors HER1/EGFR, HER2, HER3 and HER4 are a family of receptor tyrosine kinases that activate intracellular signalling pathways in response to extracellular signals[93]. The receptors consist of an extracellular domain that binds ligands, a transmembrane region, an intracellular kinase domain and an intracellular c-terminal tail[93, 94]. When ligands bind to the extracellular domain, the receptors form homo- or heterodimers with other HER family members[94], with the exception of HER2 which is not ligand-regulated[95]. The HER tyrosine kinases respond to the dimerisation by phosphorylation of the c-terminal tyrosine residue (although HER3 has a severly impaired tyrosine kinase activity[93, 96]), thus activating intracellular signalling cascades. The two major signalling cascades are KRAS-v-RAF murine sarcoma viral oncogene homologue B1 (BRAF)- mitogen-activated protein kinase kinase (MEK)-mitogen-activated protein kinase (MAPK) and phosphatidylinositol-4,5-bisphosphonate 3-kinase (PI3K)- protein kinase B (AKT) which both play an important role in gene regulation, leading to cellular responses involving cell proliferation, differentiation, migration and survival[94, 95, 97, 98]. An illustration of the EGFR signalling pathways is seen in Figure 5.

The HER family receptors are expressed in different types of normal tissue, epithelial, mesenchymal as well as neuronal[97], and are essential for normal cell function[97]. However, abnormal activation of these receptors, e.g. by ligand binding, overexpression or mutation, is deeply involved in the pathogenesis of several solid tumours[99-101]. Thus, HER receptors offer ideal targets for cancer

treatment, and have also been suggested as prognostic markers[100, 102-104]. While EGFR and HER2 have successfully been exploited as targets of therapeutic intervention, with survival benefits in selected cases of breast, colon, gastric and lung cancer[105], no Ras proteins have yet yielded to therapeutic attack[106].

In this thesis, the IHC expression of EGFR, HER2 and HER3, the mutational status of EGFR and gene copy alterations of HER2, have been examined in tissue from Cohort 1. The IHC expression of EGFR, HER2 and HER3 has been examined in tissue from Cohort 2.
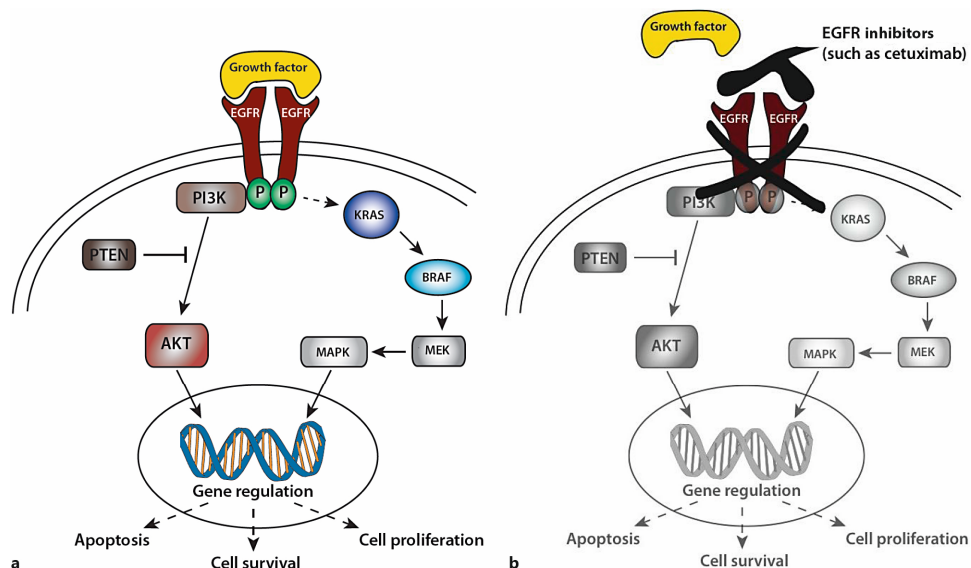
Figure 5
Illustration of the EGFR signalling pathway. (a) Binding of ligands, typically growth factors, causes dimerisation of the epidermal growth factor (EGF) receptors, which activates the pathway by autophosphorylation of the intracellular receptor tyrosine residues. The phosphorylated receptors lead to further activation of two major signalling cascades: the KRAS-BRAF-MEK-MAPK and the PI3K-AKT. Both play an important role in gene regulation, leading to cellular responses involving apoptosis, cell survival and proliferation. (b) EGF receptors are frequently expressed in epithelial tumours, and the use of EGFR inhibitors, blockning the signalling cascade, has been shown to be an important addition in modern cancer treatment. Reproduced with permission from S.Karger AG, Basel[98].

EGFR

EGFR was the first receptor shown to be overexpressed in cancer[105], and it was the first of the HER-family members for which a successful blocking agent was developed and approved for clinical use[96]. EGFR blocking is now used in the treatment of non-small cell lung cancer but no anti-EGFR therapy has been successful in the treatment of esophageal or gastric cancer to date[61, 63, 66, 67]. Structural alterations as well as overexpression of EGFR are seen in many cancer

types[96, 101], but EGFR mutations appear to be rare in esohageal and gastric cancer[107, 108]. Overexpression of EGFR in gastric cancer ranges from 2-27%, possibly due to non-standardised EGFR testing methods[107, 109]. Although not being completely unanimous, most studies indicate EGFR overexpression to be an adverse prognostic factor in gastric cancer[109].

HER2

The most common mechanism of activation of HER2 is through gene amplification, which leads to overexpression of the HER2 protein, but gene mutations can also lead to its activation[109]. An inherent correlation to prognosis has not been proven in esophageal or gastric cancer (although overexpression confers a worse prognosis in breast cancer)[62, 109, 110], but blocking of the HER2 receptor has been demonstrated to confer a significantly improved overall survival for patients with advanced gastric or gastroesophageal junction tumours overexpressing the receptor[62]. HER2 overexpression rates in gastric cancer vary widely in the literature, partly depending on technical issues[109].

HER3

Although HER3 has a severly impaired intrinsic tyrosine kinase activity, making heterodimerisation with other HER family members essential[97], it has been shown to function as a (possibly HER2 dependent) oncogene via its ability to activate PI3K signalling by binding directly to the regulatory site of PI3K, which is unique within the HER family[93]. In spite of increased expression in many cancer forms, HER3 has so far not been proven to be an adverse prognostic factor[94]. To date, no drugs targeting HER3 exist[61], however the possible role of HER3 in resistance to EGFR and HER2 inhibitors is under investigation[93].

KRAS

KRAS is a proto-oncogene that lies downstream of EGFR as part of the KRAS-BRAF-MEK-MAPK pathway[3, 98]. All carcinogenic mutations of the KRAS gene affect the guanosine triphosphate (GTP)-binding domain, decreasing its hydrolysing activity, which results in a permanently active protein that permits the cell to evade apoptosis[3]. Activating mutations in KRAS are common in e.g. colorectal cancer[111], but have also been reported in 4-16 % of gastric carcinomas[63, 107, 112]. A KRAS mutation makes the tumour ineligible for anti-EGFR therapy, but has not been demonstrated to be prognostic in upper gastrointestinal cancer[63, 112]. In this thesis, the mutational status of KRAS codons 12, 13 and 61 has been examined in the tumours from Cohort 1.

The present investigation

Aims

The main objective of this thesis was to compile two well-characterised cohorts of patients with adenocarcinoma in the esophagus or stomach with known clinical outcomes: one encompassing patients who had not received neoadjuvant treatment, and one encompassing patients who had received neoadjuvant treatment. Tumour tissue microarrays were then constructed in order to study the expression of potentially prognostic or treatment predictive proteins.

A specific aim was to examine the expression and prognostic significance of SATB1 and SATB2 in primary tumours and lymph node metastases, as this had not been studied previously in esophageal cancer and only to a very small extent in gastric cancer.

Another specific aim was to study potential changes in the tumour-specific expression of EGFR, HER2 and HER3 after neoadjuvant therapy and correlate this with survival, which has not been described before. In addition, we examined the prevalence and prognostic impact EGFR, HER2 and HER3 as well as alterations of HER family protein expression from primary tumours to lymph node metastases.

Material and Methods

Patients

Cohort 1

This is a consecutive cohort, originally encompassing a total of 303 patients with esophageal or gastric adenocarcinoma, who had undergone surgery at the University hospitals of Lund and Malmö from January 1st 2006– December 31st 2010. Haematoxylin & eosin slides were examined and after exclusion of cases having received neoadjuvant treatment, being mucosal resections or having double or incorrect classification or missing specimens, there were 175 remaining cases. From these, tissue was selected from the primary tumours, lymph node metastases and tumour-adjacent benign tissue. After publication of Paper I, it was discovered that one of the 175 patients had received neoadjuvant treatment, and this patient was therefore excluded from further analysis. Tumour stage was classified according to the TNM7 classification[113]. Clinical data, information on recurrence, vital status and cause of death were obtained from the medical charts. A flow-chart of the patients in Cohort 1 is provided in Figure 6.

Figure 6
Patients and tissue in Cohort 1

Cohort 2

This is a consecutive cohort of 166 patients with esophageal or gastric adenocarcinoma, who had received neoadjuvant treatment according to current Swedish national guidelines[15] at the University hospitals of Lund and Malmö during the period January 1st 2008– December 31st 2014. Haematoxylin & eosin slides were examined and material from pre-treatment biopsies, surgical resections including tumour-adjacent benign tissue, lymph node and distant metastases as well as recurrences was selected for all available cases. Three cases lacked sufficient tissue, and from the remaining 163 cases biopsy tissue was available in 159 cases and tissue from surgical resections in 116 cases. Tumour stage was classified according to the TNM7 classification[113]. Pathological response to treatment according to Chirieac[5] was evaluated on the surgical resection specimens. Clinical data, data on neoadjuvant treatment, information on recurrence, vital status and cause of death were obtained from the medical charts. A flow-chart of the patients in Cohort 2 is provided in Figure 7.
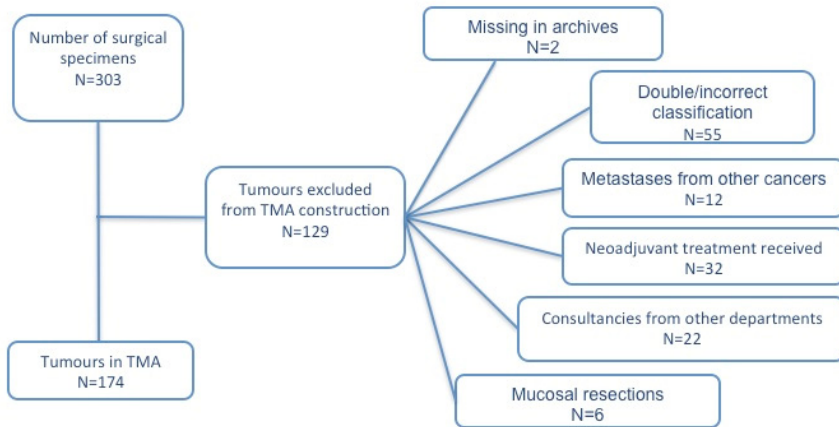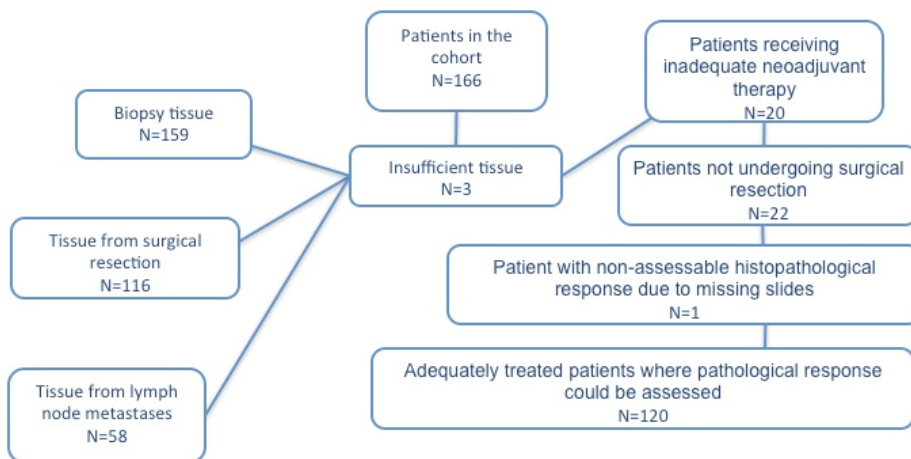
Figure 7
Patients and tissue in Cohort 2.

Tissue microarray technology

A tissue microarray (TMA) is a paraffin block containing multiple donor tissue cores. The cores, usually 0.6 – 2 mm in diameter, are taken from formalin-fixed, paraffin embedded different parts of a tumour or from different tumours or tissues. Sections are cut from the TMA block and subjected to examination, thus enabling high-throughput simultaneous in-situ detection of DNA or protein expression using only a fraction of antibody and tissue material compared to analysis of full-face tissue sections[114]. Since its introduction in 1998, the technique has become an important tool in biomarker research[115]. One caveat concerning this technique is that because many tumours are heterogeneous, very small tissue samples may not always reflect the biological properties of the entire tumour[114, 116, 117]. This can, at least in part, be compensated for by using more than one tissue core from each tumour, ideally from different areas of the tumour, and by verification of the TMA results by analysis of larger tissue specimens before clinical application[114]. Furthermore, even with the use of full-face sections, sampling bias is not excluded, as these also represent only a limited fraction of the tumour. An advantage of the TMA approach is the high number of tumours that can be studied simultaneously, which conceivably might compensate for false negative or false positive tissue cores[118]. Figure 8 illustrates the construction of a TMA.
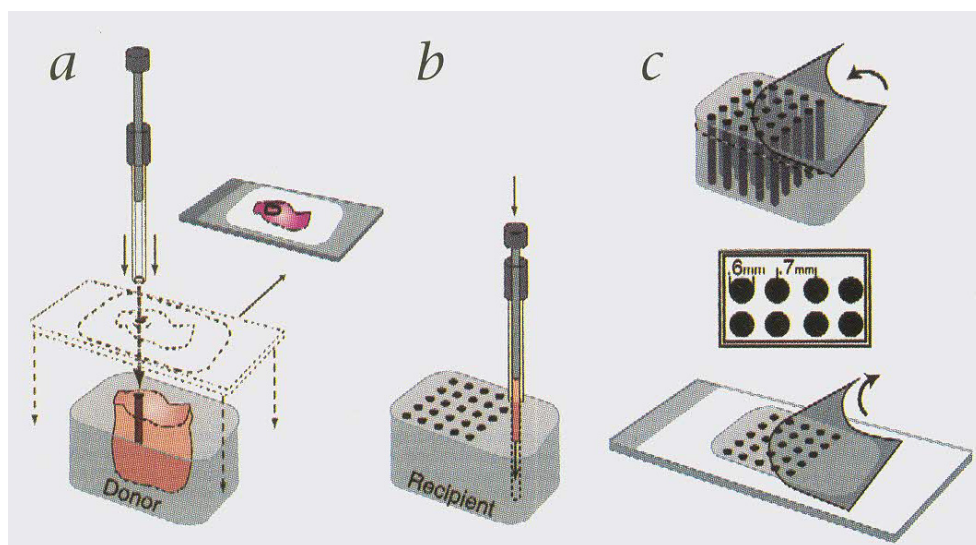
Figure 8
Construction of a tissue microarray. Cores taken from formalin-fixed, paraffin embedded donor tissue are arranged in in a recipient paraffin block, from which multiple sections can be made. Reprinted with permission from Nature Publishing Group[114].

In this thesis, paraffin blocks with TMAs were constructed using 1 mm cores of non-necrotic tissue from primary tumours, lymph node metastases, recurrences, intestinal metaplasia (IM) and benign tissue. Duplicate cores were, whenever possible, obtained from different blocks of the primary tumour and from different lymph node metastases. Pre-treatment biopsies were represented in full-face sections.

Immunohistochemistry

Endeavours to trace proteins or antigens in tissue have been ongoing for more than 100 years[119]. In the 1940's Dr Albert Coons introduced immunohistochemistry (IHC), i.e. the use of colour-tagged antibodies to localise antigens in tissue using fluorescence microscopy, and Nakane further developed the technique and made it possible to see the reactions in a light microscope[115]. IHC has now been used extensively in diagnostic pathology for more than 40 years and is essential for the diagnosis and sub-classification of many neoplastic lesions, since the results increasingly contribute to the prognostication and choice of treatment for patients[120]. The technique allows not only for identification of the antigen in a morphological context of the cell, but also gives an indication of its function in

vivo, as opposed to other molecular assays such as DNA sequencing or messenger-ribonucleic acid (mRNA) analyses[115].

When tissue has been removed from a patient, a fixation process needs to be started as soon as possible in order to avoid autolysis[15]. The most common method is fixation in formalin[121, 122]. Formalin binds to the proteins of the tissue, creating methylene bridges and stabilising the tissue[15], but also causes conformational changes of protein epitopes[122]. Thereafter, the tissue is dehydrated, embedded in paraffin and then cut into 3-7 µm sections. For IHC purposes, the epitopes need to be "demasked" for the antibodies to be able to bind to them, which is why antigen retrieval, a partial reversal of fixation in order to let the epitopes regain their original conformation, is performed[122]. Then the antibody can be applied, either directly by use of a primary antibody only or indirectly using a primary antibody that binds to the epitope and then a secondary antibody that binds to the primary antibody, and visualises it by means of enzymes that form a colour, visible in light microscope, when a chromogen is added[122]. The primary antibody is either mono or polyclonal, the monoclonal type comprising antibodies against only one antigen epitope, and the polyclonal type recognising many epitopes of the same antigen[115].

Many parameters of these processing steps affect the specificity and sensitivity of the IHC method[120-122]. Choice of antibody and interpretation of the reaction have been demonstrated as the most important factors for diagnostic outcome[115], and the need for better standardisation and quality control in this process has been emphasised[120, 121]. Illustrations of the effects on the result, which suboptimal methods and material can have are seen in Figures 9 and 10.
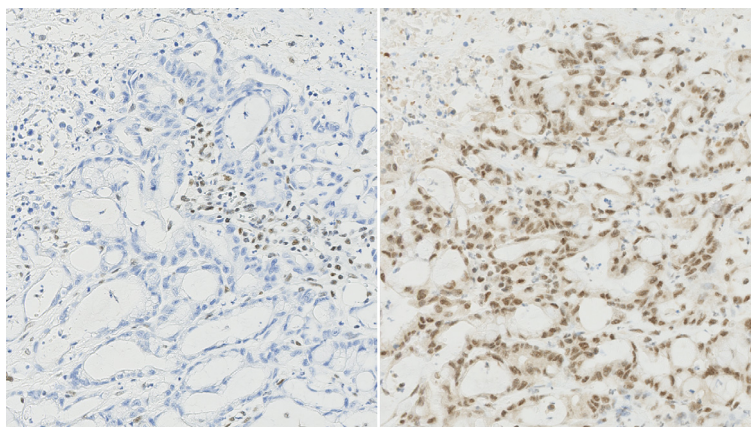
Figure 9
An illustration of how different antibodies can yield different results. The antibody clone directed against a mismatch repair protein (mutL homologue 1, MLH1) used in the picture to the left correctly yields a negative staining of the neoplastic cells revealing a mutation in the tumour, whereas the clone used in the picture to the right yields a false positive staining. Reproduced with permission from Prof M Vyberg, NordiQC.
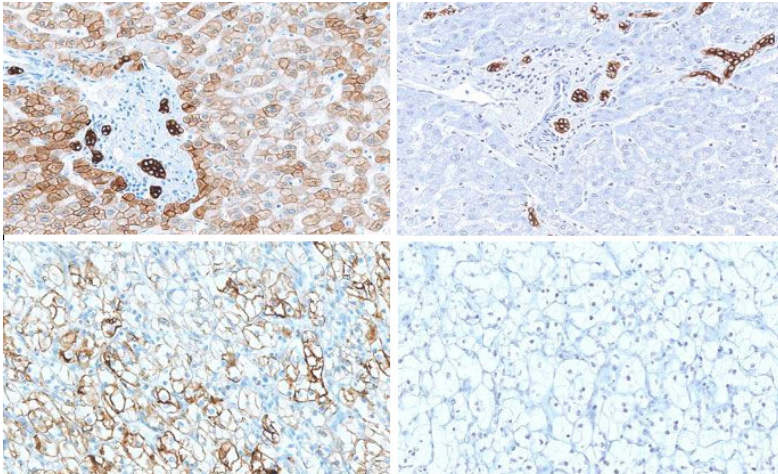
Figure 10.
An illustration of how different methods of antigen retireval yield different results. In the upper row, liver tissue stained for cytokeratins is shown. Proper demasking of the antigen used in the picture to the left shows the correct staining of the liver cells. To the right, a false negative reaction with the same antibody but without proper demasking of the antigen is shown. In the lower row, a renal cell carcinoma is shown, giving a correct staining to the left but a false negative reaction to the right, due to the poor protocol. Reproduced with permission from Prof M Vyberg, NordiQC.

In this thesis, the expression of SATB1, SATB2, p53, Ki67, EGFR, HER2 and HER3 have been evaluated by IHC and the specificitity of the anti-SATB2 antibody AMAb90679 CLO320 and the anti-HER3 antibody SP71 were demonstrated partly by use of IHC.

In situ hybridisation

In situ hybridisation (ISH) allows for the direct quantification of gene copy number per nucleus by the use of a DNA probe labelled with a fluorescent, chromogenic or silver detection system (fluorescence in situ hybridisation (FISH), chromogenic in situ hybridisation (CISH) or silver in situ hybridisation (SISH), respectively), complementary to the target DNA sequence[123]. In this thesis, SISH, offering the advantage of detection via light microscope, was used for the evaluation of HER2 gene copy alterations. The reaction can be carried out on formalin-fixed, paraffin embedded material and begins with the denaturation of DNA through enzyme digestion. Labelled probes for the HER2 and chromosome 17 are then added, the two target sequences are cohybridised and then detected in their morphological context via light microscopy on hematoxylin-counterstained slides[123, 124].

In this thesis, IHC stains were performed on 4 μm sections of the TMA-blocks and on 3 μm sections from the pre-treatment biopsies. For immunocytochemistry, cultured cells were harvested and spun down to cell pellets, which were paraffin embedded and cut into sections. Different IHC and ISH staining protocols and models of assessing the stainings were used for the different antibodies in accordance with current guidelines and best practises[41, 46, 47, 62, 71, 75, 100, 125-129].

Pyrosequencing

Pyrosequencing is a means of determining the order, or sequence, of nucleotides in short strands of DNA[130, 131]. The reaction begins with a template DNA strand, to which a polymerase (that synthesises DNA) is added. Nucleotides are injected into the pyrosequencing chamber one at a time. When a correct complementary nucleotide is injected it is incorporated by the polymerase. Through this reaction, a pyrophosphate molecule is released, which is then converted into adenosine triphosphate (ATP) and later into light. The emission of light indicates that the inserted nucleotide has been incorporated and as the order of the injected nucleotides is known, the sequence of the DNA template can thus be determined[130, 131]. In this thesis, pyrosequencing was used to analyse EGFR and KRAS mutation status, as described in Paper III.

Small interfering RNA transfection

Small interfering ribonucleic acids (siRNAs) offer a method of sequence-specific silencing of a specific gene without genomic manipulation and has become an important tool for understanding gene function[132]. siRNAs are synthesised strands of ribonucleic acid (RNA) that, when introduced (transfected) into the cytoplasm of a cell, can bind a complementary mRNA strand and cleave it, thus inhibiting translation of the protein encoded by the targeted mRNA[132-134]. In this thesis, siRNA transfection was used for silencing of the HER3 protein, in order to demonstrate the specificity of the SP71 HER3 antibody.

Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) is used to measure DNA amplification as it occurs, making it possible to determine the relative concentration of DNA[135]. The reaction begins with a heat-induced melting of the double-stranded DNA of a sample into single strands. Heat-stable polymerase molecules then copy and amplify the gene of interest via repeated cycles of heating and cooling, with the help of nucleotide building blocks and primer DNAs complimentary to the gene of interest. The amplified DNA is fluorescently labelled and measured after each cycle, enabling calculation of the amount of DNA present at the beginning of the reaction[135].

In this thesis, qPCR was used in combination with reverse transcription PCR (rt-PCR) to detect and quantify mRNA levels to confirm silencing of gene expression by siRNA. The mRNA was converted to complementary DNA (cDNA) by reverse transcription (rt-PCR) before being used in qPCR assays.

Western blot

Western blot is a technique used to separate and identify proteins[136]. First, a lysate of cells is created, denatured and loaded on to a gel. The amino acids of the proteins now have a negative charge and travel through the gel towards a positive electrode when voltage is applied. The semi-porous quality of the gel makes smaller proteins travel faster, and the proteins are thus separated based on molecular weight. The proteins are then transferred to a membrane and an antibody applied to detect the protein of interest. A secondary, enzyme-conjugated antibody is added, which can convert a substrate into a signal detected by chemiluminescence, visualising the protein[136]. In this thesis, Western blot was used to assess the specificity of anti-SATB1 and anti-SATB2 antibodies as described in Paper I.

Statistical analyses

For analysis of the relationship between the investigated biomarkers and clinicopathological parameters, the non-parametric Mann-Whitney U test was applied for continuous variables and the chi-squared test for categorical variables. For assessment of changes in HER protein expression in pre-treatment biopsies compared with post-treatment tissue, the non-parametric Wilcoxon matched pairs

test was used. To illustrate survival probabilities, stratified by the variables of interest, Kaplan-Meier graphs were applied. To display the statistical uncertainty of the Kaplan-Meier graphs, life tables were added. To assess differences in the Kaplan-Meier curves, the log-rank test was used. Cox regression analyses were performed to confirm differences in survival between groups, and hazard ratios for death and recurrence were calculated. The Cox regression analyses were made both without taking the influence of other factors into account (univariable analysis) and with taking the influence of selected parameters that may affect survival into account (multivariable analysis).

For paper I, IBM SPSS Statistics version 20.0 (IBM Corporation, Armonk, NY, USA) was used. For papers II-IV, version 22.0 was used. P-values <0.05 were considered statistically significant. All tests were two-sided. Statistical methods were chosen according to the properties of the material and in accordance with common practise in medical statistics, where Kaplan-Meier graphs, log-rank tests and Cox proportional hazard models are the three most commonly used methods for survival analyses in cancer journals[137-139].

Summary of results and discussion

SATB1 and SATB2

Initially, Western blot analysis of SATB1 and SATB2 overexpressing mammalian cells and IHC staining of rectal and tonsil tissue validated the specificity of the investigated anti-SATB1 and anti-SATB2 antibodies.

The results in Paper I demonstrated SATB1 to be expressed in 31.2% of the primary tumours and in 40.5% of the metastases, whereas no expression was seen in benign tissue at all. Furthermore, SATB1 expression was an independent adverse prognostic factor in radically resected tumours (hazard ratio (HR)=2.30; 95% confidence interval (CI) 1.24-5.16). These results augment the evidence that SATB1 can promote aggressive cell behaviour. SATB1 expression was also associated with younger age and more advanced N-stage, and was more common in esophageal than in gastric tumours. The difference in SATB1 expression seen between primary tumours and metastases was not statistically significant. SATB1 expression was higher in IM than in normal tissue, but the number of samples was small. SATB1 expression was significantly lower in primary tumours with tumour-associated IM than in tumours without tumour-associated IM, and patients with tumour-adjacent IM had a trend, however non-significant, towards a longer overall survival (OS) than patients with tumours without tumour-adjacent IM. These findings confirm SATB1 expression as a negative prognostic factor and the existence of at least two different pathways of gastroesophageal carcinogenesis, one intestinal (arising from dysplasia in IM) and one non-intestinal, the former being associated with a better OS[20]. Considering the role of SATB1 as an organizer and global regulator, the associations found in this study could be considered weak. However, the exact function of SATB1 in cancer cells is not clear. As an example, the structural organization of SATB1 has been suggested as more important for an aggressive phenotype than level of expression, in a study of prostate cancer cell lines[140, 141]. Further studies are warranted to understand how SATB1 affects cancer cells. Figure 11 shows an example of an esophageal adenocarcinoma expressing SATB1.
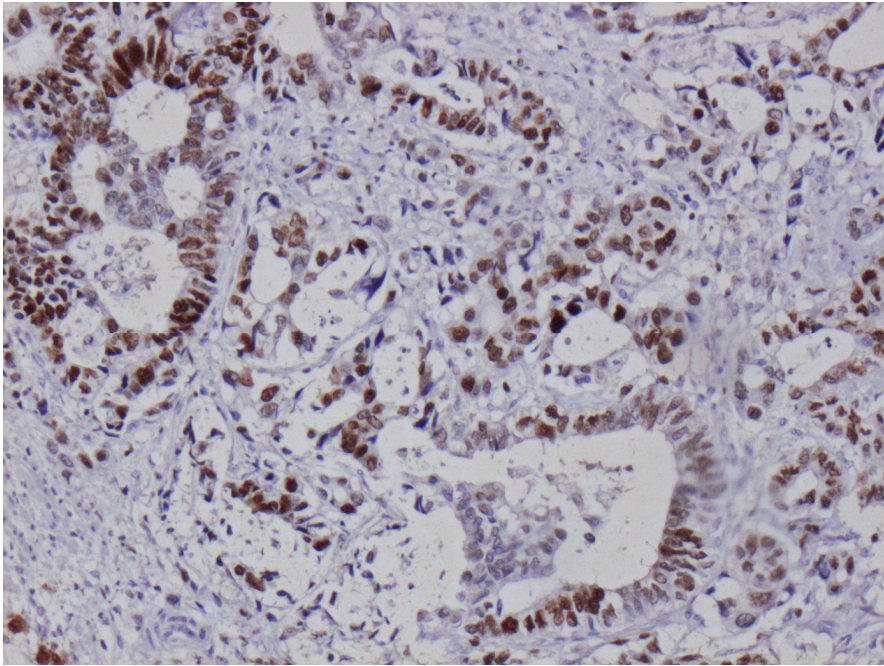
Figure 11
Example of an esophageal adenocarcinoma expressing SATB1.

Since SATB2 was expressed to a very limited extent in the examined tissues and no correlation to OS was seen, no further statistical analyses were performed. These results are in line with a previous study indicating SATB2 expression to be relatively restricted to cancer cells of colorectal origin[82].

Ki67 and p53

Neither Ki67 nor p53 expression were found to be correlated to OS of the patients in Cohort 1. Regarding p53, these results are consistent with previous studies of upper gastrointestinal cancer that have not proven any certain impact of p53 expression on survival[87, 142, 143], although an association with shorter OS has been indicated[144]. According to a recent meta-analysis, previous studies on the prognostic impact of Ki67 expression in esophageal cancer have not demonstrated any significant associations with OS[87]. Equally, no prognostic impact has been proven for Ki67 expression in gastric cancer[145], which is in line with the results from this thesis. However, Ki67 is understudied in esophageal cancer and a lack of adverse prognostic influence could be due to study design or sample size.

KRAS

KRAS mutations were seen in 4.1% of the tumours from Cohort 1, without any association with OS, as demonstrated in Paper III. The percentage of KRAS-mutation is concurrent with previous findings in esophageal and gastric cancer[63, 107, 112]. Because of the low percentage of KRAS-mutated tumours, the sample size is too small for any further conclusions to be drawn from this study.

HER family

Standard care for patients with esophageal or gastric adenocarcinoma includes neoadjuvant treatment with chemotherapy or chemoradiotherapy. Many patients are also treated with targeted therapy against the HER2 receptor in the palliative setting[57, 58, 62]. Very few studies have investigated if neoadjuvant therapy invokes a change in the expression of HER family proteins[146]. Such studies are however of importance, as positive conversion may affect treatment strategy. There are currently no recommendations regarding which type of specimen, i.e. pre-treatment or post-treatment, is preferable for biomarker analysis. Another aspect of conversion of expression is the potential prognostic impact of a conversion *per se,* which has not previously been extensively investigated. In line with the results from papers III and IV, a few other studies have indicated primary-metastatic conversion, or intratumoural heterogeneity, speculatively mirroring an unstable genome, to be an adverse prognostic factor[36, 38]. Figure 12 shows sample images of EGFR, HER2 and HER3 expression.
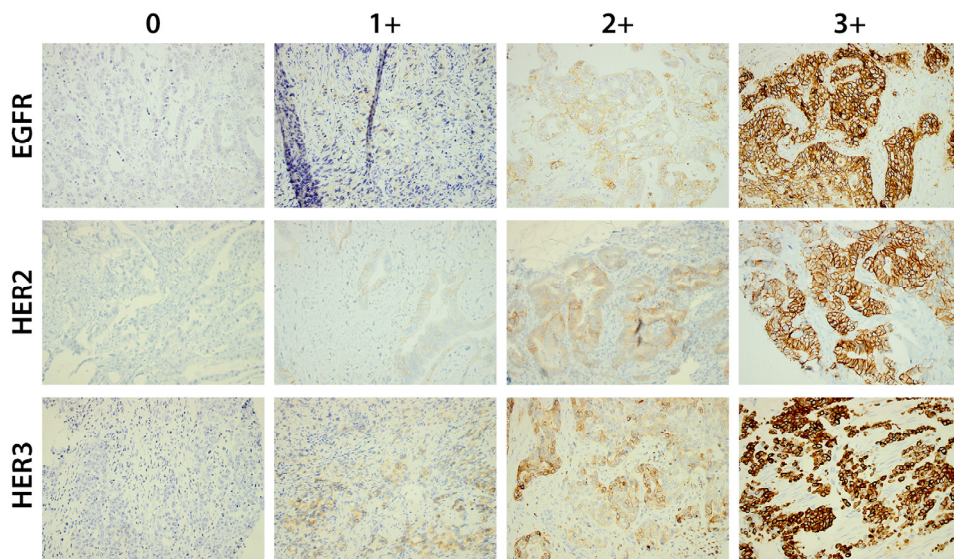
Figure 12
Sample images of EGFR, HER2 and HER3 expression displaying score 0, score 1, score 2 and score 3.

EGFR

As demonstrated in papers III and IV, expression of EGFR in tissue from Cohort 1 and 2 was higher in malignant than in benign tissue, although the differences were not statistically significant. High EGFR expression was seen in 4.7% of the tumours from Cohort 1 and in 7.1% of the pre-treatment biopsies from Cohort 2, which is in agreement with findings from previous studies, although reported rates of EGFR overexpression vary widely[95, 147-149]. There were no significant associations between IHC expression of EGFR and clinicopathological parameters in tumours from Cohort 1 (paper III). Paper IV demonstrated a significant association between high EGFR expression and more advanced clinical T-stage in tumours from Cohort 2 ($p = 0.017$) and high EGFR expression was demonstrated to be more frequent in the esophageal compared with the gastric tumours ($p = 0.016$).

In line with the majority of reports on the prognostic impact of EGFR expression in esophageal and gastric adenocarcinoma[103, 104, 149, 150], Kaplan-Meier analysis of EGFR expression in the tumours from Cohort 1 revealed high EGFR expression to be associated with shorter OS. The prognostic value of EGFR was confirmed in unadjusted as well as in adjusted Cox regression analysis (HR = 2.42; 95% CI 1.18–4.96, $p = 0.016$ and HR = 2.42; 95% CI 1.16–5.07, $p = 0.019$, respectively). In cohort 2, Kaplan-Meier analysis demonstrated no significant

impact of pre-treatment biopsy or post-treatment surgical resection protein expression on OS (*p*= 0.486 and *p*=0.248, respectively). To date, no classification system has been implemented for the assessment of EGFR status in esophageal or gastric cancer. We have chosen to use the guidelines of gastric HER2-scoring, which are well validated for HER2 evaluation and most likely adequate for EGFR scoring as well, although other studies have used other scoring systems[151, 152], and it can not be ruled out that some other scoring system may better reflect the prognostic impact of EGFR.

As discussed above, many aspects of biomarker investigation may potentially affect the results. The lack of consistent and validated methods and testing algorithms in investigational as well as clinical studies of EGFR and HER3, and to some extent also HER2, may lead to a lack of consistent results. The need for validated methods and testing algorithms regarding EGFR and HER3 specifically has been stressed in previous studies[100, 149, 153].

None of the tumours from Cohort 1 were EGFR mutated, which is in accordance with previous studies, suggesting EGFR mutations to be rare in esophageal and gastric adenocarcinomas[63, 107, 108, 153].

When a cancer becomes metastatic, heterogeneity may become more pronounced and biological properties of metastatic tumours may differ considerably from the primary tumour, possibly mirroring an unstable genome prone to acquiring new mutations supporting its survival. Concordance studies on the expression of different growth factor receptors in primary and metastatic tumours have previously been performed in several types of cancer, including gastric cancer. However, most of these studies did not investigate whether discordance had any prognostic impact[36, 48-51]. Conversion of EGFR expression from high to low, or from low to high, between primary tumour and lymph node metastases in Cohort 1 (seen in 8 cases) had no prognostic impact. Only one case of conversion between primary tumour and paired lymph node metastasis was seen in Cohort 2.

Conversion of EGFR expression in pre-treatment biopsies compared with post-treatment surgical resections was seen in 4.6% (n = 5) of the cases in Cohort 2, and this was associated with a trend toward a shorter OS (*p* = 0.064).

The observed adverse prognostic impact of EGFR expression in gastric and esophageal tumours suggests a potential for drug targeting. Several monoclonal antibodies as well as tyrosine kinase inhibitors have been evaluated in different settings for metastatic esophageal and gastric cancer, but without significant benefit[95, 107, 153-156]. The reason why EGFR inhibition has been far less beneficial in esophageal and gastric cancer than in e.g. colorectal and lung cancer is not known and requires further study. One explanation could be the creation of compensatory pathways in response to inhibition of HER family receptors, and

one way forward may therefore be combination strategies targeting more than one HER family member[95, 157].

There was no significant association between EGFR expression and histo-pathological response in the tumours from Cohort 2.

HER2

In paper II, HER2 expression in the tumours from Cohort 1 was investigated using IHC and SISH, with a 91.4% concordance rate between IHC and SISH, which is consistant with previous studies[45]. HER2 overexpression was denoted in 18.% of the tumours, according to a combined overexpression variable (IHC3+ and/or SISH+), and 3+ protein expression was seen in 9.5% of the pre-treatment biopsies from Cohort 2. Both findings are in accordance with previous studies with HER2 overexpression rates ranging between 7 and 34%[45, 62]. Protein expression as well as gene amplification was significantly associated with higher differentiation ($p = 0.002$ and $p = 0.002$) and intestinal morphology ($p = 0.003$ and $p = 0.005$) in the tumours from Cohort 1, which is in agreement with previous findings[26, 62, 158, 159]. The same associations were seen when protein expression of HER2 was examined in the tumours from Cohort 2 ($p = 0.007$ and $p = 0.004$). Protein overexpression, but not gene amplification, was more frequent in esophageal compared with gastric location in the tumours from Cohort 1 ($p = 0.003$).

Whether the amplification or overexpression of HER2 in gastric cancer specimens has any inherent prognostic impact is still unclear[42, 44, 159]. HER2 overexpression in the tumours of Cohort 1, protein overexpression in the pre-treatment biopsies or protein overexpression in the post treatment surgical resections from Cohort 2 had no impact on OS.

Intratumoural heterogeneity has long been recognised as a potentially malignant feature[36, 38, 39], and it is fairly well established that heterogeneity is more common in gastric carcinoma than in for example breast cancer[45, 46]. However, very few studies have addressed the prognostic significance of intratumoural heterogeneity in gastric cancer[36, 39]. In this study, intratumoural HER2 heterogeneity of the tumours from Cohort 1 had no impact on survival.

Conversion between primary tumours and paired lymph node metastases according to the combined HER2 overexpression variable was seen in 12.9% of the cases in Cohort 1, with concordance between TMA cores and full-face sections in 7 of 9 cases. Conversion was significantly associated with shorter OS in both unadjusted (HR = 2.14; 95% CI 1.00–4.57, $p = 0.049$) and adjusted (HR = 4.93; 95% CI 1.96–12.39, $p = 0.001$) Cox regression analysis. This association remained significant when cases with negative conversion were excluded, when the two

cases without concordance between TMA and full-face sections were excluded, and when intratumoural heterogeneity was included in the analysis. In Cohort 2, only one case displayed conversion between the primary resection specimen and paired lymph node metastasis. This patient had a shorter OS compared to patients without conversion ($p = 0.049$).

Conversion of HER2 expression from pre-treatment biopsies to post-treatment surgical resection specimens was seen in 6 (5.9%) cases in Cohort 2, of which 5 were a conversion from high expression to low. This conversion had no prognostic impact ($p = 0.878$). Watson *et al* saw a similar downregulation of HER2 expression after chemotherapy and speculated whether this could be due to a higher chemosensitivity in HER2-positive tumour cells, which could, speculatively, be the case in this study as well[146].

No significant association was seen between HER2 protein expression and histopathological response in the tumours from Cohort 2.

Laboratory techniques and choice of antibody have been shown to have a significant influence on the interpretation of HER2 overexpression[160]. Furthermore, concordance of HER2 protein expression and gene amplification in gastric carcinoma is controversial, as is the subject of which method is the best to evaluate overexpression with the aim to predict therapy response, protein overexpression or gene amplification[44, 47, 62, 125]. Therefore, our stainings were performed in a laboratory with experience of gastric HER2 staining, using a well-validated antibody recommended by Rüschoff[125]. In addition, all cases in Cohort 1 were subjected to examination of HER2 protein expression as well as gene copy number, which must render our results credible.

HER3

Initially, qPCR analysis and immunocytochemical staining of human gastric adenocarcinoma cells that had undergone siRNA mediated knockdown validated the specificity of the investigated anti-HER3 antibody.

Similarly to EGFR, and as demonstrated in paper III and IV, expression of HER3 in tissue from Cohort 1 and 2 was shown to be higher in malignant than in benign tissue, although the differences were not significant. High HER3 expression was seen in 23.8% of the primary tumours from Cohort 1 and in 22.6% of the pre-treatment biopsies from Cohort 2, which is in line with previous findings in upper gastrointestinal cancer, although reported rates of HER3 overexpression vary extensively[95, 102, 128, 147]. There were no significant associations between IHC expression of HER3 and clinicopathological parameters in Cohort 1 or 2.

In Cohort 1, KM analysis demonstrated that patients with tumours expressing high levels of HER3 had a prolonged OS, although this difference was only significant between tumours with high (3) and negative (0) expression. The positive prognostic impact of high HER3 expression was borderline significant in unadjusted, but not in adjusted Cox regression analysis in Cohort 1 (HR = 0.65; 95% CI 0.41–1.04, p = 0.052 and HR = 0.92; 95% CI 0.57–1.48, p = 0.732, respectively). HER3 pre-treatment biopsy protein expression had no significant impact on OS in Cohort 2 (p = 0.664), but high expression after neoadjuvant treatment was significantly associated with a longer OS in KM analysis (p = 0.027) and in univariable (HR=0.39; 95% CI 0.17-0.93, p = 0.033), but not in multivariable (HR=0.43; 95% CI 0.17-1.07, p = 0.069) Cox regression analysis. Other studies have demonstrated associations between HER3 expression and both shorter and longer OS in several cancer types[100, 102, 147, 161, 162]. Since a prognostic impact for HER3 expression has been very difficult to ascertain, any such impact is likely to be of minor clinical importance, and perhaps further investigational efforts should be focused on HER3 as a potential drug target instead of as a prognostic biomarker.

Conversion between primary tumour and lymph node metastases in tumours from Cohort 1 was seen in 4 cases (all low to high) and had no prognostic impact. Conversion of HER3 expression between primary resection specimen and lymph node metastasis was seen in 7 (18.4%) cases in Cohort 2, without any significant association with OS.

Conversion of HER3 expression in pre-treatment biopsies compared with post-treatment surgical resections was seen in 19 (19.4%) of the cases in Cohort 2, with positive conversion in 12/19 cases. HER3 conversion was not significantly associated with OS (p = 0.569).

The finding of a greater proportion of high HER3 expression in post-chemotherapy tissue, compared with pre-chemotherapy tissue is new. This has previously only been investigated in relation to targeted HER-therapy, whereby HER3 was found to be upregulated in response to HER-inhibition[94, 157]. Since HER3 has been indicated to function as a signalling hub for the HER family, leading to compensatory pathways via its upregulation, thereby promoting resistance to multiple therapeutic agents[94, 97, 157, 163, 164], it is plausible to assume that chemotherapy treatment may invoke an upregulation of HER3. Therefore, HER3 may also be a potential drug target. Monoclonal antibodies against HER3 have been examined in preclinical settings as well as clinical trials e.g. for breast- and colorectal cancer[95, 97], but to date, no HER3-targeting drugs are in clinical use. One possible reason for HER3 being upregulated in tumours but not having any clear impact on OS could also be that it is expressed in non-proliferating parts of a tumour, as has been shown to be the case in benign colonic

crypts[165]. This could also shed light on why HER3-targeting drugs have been unsuccessful so far, theoretically contributing to a diminishing tumour bulk, but not affecting the proliferative cancer stem cell like population.

No significant association was seen between HER3 expression and histo-pathological response in the tumours from Cohort 2.

Histopathological response

Histopathological response was evaluated in the surgical resection specimens from Cohort 2 and denoted into four semi-quantitative categories according to Chirieac[5]. Significant associations were seen between histopathological response and OS as well as time to recurrence (TTR), with the group with no residual carcinoma having the best prognosis and the group with more than 50% residual carcinoma having the worst prognosis. For OS, the difference between the group with no residual carcinoma and the group with >50% residual carcinoma was significant ($p = 0.008$), and for TTR, the prognostic difference was significant for both >50% and 11-50% residual carcinoma, as compared to no residual carcinoma ($p = 0.001$ and $p = 0.025$, respectively). These associations not only validate this method as an important prognostic tool[5], but also bring to mind its potential use as a guide when deciding post-surgery treatment strategy. For example, one study demonstrated that in a group of patients with ypT3 esophageal tumours there was a significant prognostic difference between patients with less than, as compared to more than, 50% residual tumour[14]. This information may be relevant when individualised risk-adopted post surgical aftercare is to be determined

Three-tiered regression scoring systems have been preferred in some studies due to a higher reproducibility with high inter-observer reliability[14]. However, as scoring of all tumours was done by one person in this study, the benefit of quantification into four categories was estimated to be larger than the potential drawback of using a system with four categories.

Strengths and limitations

In addition to the strengths and limitations discussed in Results and discussion above, a few further aspects require attention. A strength of the thesis is that both study cohorts were consecutive, ruling out any risk for selection bias regarding the included cases.

A potential caveat is the use of the TMA technique, whereby, as previously discussed, the representativity of the tissue cores in relation to whole tissue sections may be questioned. This makes it impossible to completely rule out that tissue heterogeneity may, at least in part, explain the observed differences. Other studies have indicated the TMA technique to partly overlook gastric HER2 positivity[166]. However, it must be pointed out that full-face sections also represent only a fraction of the tumour, and that the TMA technique allows for sampling from different regions of the tumour, thus enabling detection of heterogeneous expression. If one is aware of the potential pitfalls of the TMA technique and take measures to compensate for the built-in weaknesses, the TMA technique is, with some exceptions[116], a powerful and well-validated platform for large-scale tissue-based biomarker studies[118, 167]. In the construction of both TMA sets used in this thesis, two tissue cores were taken from the primary tumour and metastasis and, whenever possible, from different blocks of the primary tumour. In Paper II, the staining of HER2 was also analysed on corresponding full-face sections in 9 cases.

The immunohistochemistry technique offers an advantage in that the examined biomarker can be studied in a morphological context. This enables not only identification of the subcellular location of the protein of interest and avoidance of examination of non-malignant cells, but also gives an indication of its function *in vivo* and takes post-transcriptional processing into consideration[87, 115]. In this sense, IHC is superior to DNA or mRNA-based technologies.

The use of well-validated antibodies is, as stressed above, crucial for yielding correct results. Therefore, a further strength is that all antibodies used have either previously been demonstrated to be specific, or have been validated within this thesis[125, 127, 129]. Furthermore, we have used validated and widely used evaluation systems for IHC scoring of the investigated proteins, although no consensus criteria exist on how to best score and categorise EGFR and HER3 or SATB1 and SATB2 expression in esophageal and gastric cancer[47, 71, 100, 102,

125]. One exception from this is that the expression of HER2 in Cohort 2 has only been investigated by IHC, not by SISH, and although previous studies have indicated that the strongest predictive value is seen for protein expression and not gene amplification of HER2, this must be regarded as a limitation. Another caveat is that since location of the tumour and patient characteristics differed within the cohort, the neoadjuvant treatment was not identical for all patients.

Many tests have been performed on a limited material, which confers a risk for type I statistical errors, i.e. detection of significances that are coincidental. However, since the studies in this thesis are to some extent exploratory rather than confirmatory, the value of the studies would decrease if the numbers of analyses were decimated too much, since that would lead to an increased risk for type II errors. Therefore, we have chosen to perform the analyses we considered to be of interest, and to interpret the results with caution. Still, the small number of cases in some of the analysed groups in this thesis, such as the number of cases displaying conversion, makes the statistical analyses hazardous to interpret and further studies are warranted.

Conclusions

SATB1 is a promising prognostic biomarker in upper gastrointestinal adeno-carcinoma that merits further investigation.

EGFR expression was associated with a more aggressive tumour behaviour in some tests. HER2 expression was not significantly associated with survival. HER3 was associated with a longer overall survival in both study cohorts, although this association was not independent of established prognostic factors.

A change in HER protein expression from pre-treatment biopsy to post-treatment surgical resection was seen in 5-20% of the cases, underscoring the need for further analysis and subsequent guidelines as regards which type of tissue treatment decisions should be based upon.

A conversion of biomarker expression may be indicative of a more aggressive tumour phenotype. Speculatively, conversion may reflect genomic instability, but further studies are warranted.

It is important to use well-validated tumour material and methods in order to avoid misleading results and in order to make different studies comparable.

Histopathological response to neoadjuvant treatment was significantly associated with a longer time to recurrence as well as overall survival, and is possibly underutilised as a guiding tool when deciding post-surgery treatment strategy.

Future perspectives

The global cancer burden is increasing[1]. Althouh progress has been made regarding new treatment strategies, including traditional chemo- or radiotherapy as well as targeted drugs, improved detection techniques and more advanced surgery and supportive care, mortality rates for several cancer types remain high. Thus it is of the utmost importance to continue to strive towards a better understanding of cancer, in order to identify clinically relevant subgroups, better prognostic and predictive tools and ultimately treatment strategies.

The tumorigenesis of esophageal and gastric cancer is far from being completely understood, and esophageal cancer has been highlighted as a research priority within a plethora of unmet clinical needs[87].

Several of the findings in this thesis contribute to the ongoing efforts to elucidate the role of different HERs in cancer development and treatment, as well as the potential clinical value of the SATB1 protein. Some of the findings merit further study.

SATB1 is an interesting potential biomarker and forthcoming studies should examine its potential treatment predictive value.

The potential adverse prognostic impact of a conversion of biomarker expression needs to be validated in larger studies to enable conclusions to be drawn.

A drawback of the studies related to in paper IV is the lack of gene copy number data as well as the lack of examined full-face sections corresponding to the cases where TMA investigation indicated a change in protein expression. To strengthen our results, such full-face section analyses as well as SISH analysis of the HER2 expression are planned.

Populärvetenskaplig sammanfattning

Cancer i matstrupe och magsäck är sjukdomar som orsakar stort lidande. Magsäckscancer är den tredje vanligaste orsaken till cancerdöd i världen. I matstrupen finns både skivepitelcancer och körtelbildande cancer, i magsäcken är nästan all cancer av den körtelbildande varianten. Västvärlden har historiskt sett haft mindre förekomst av både matstrupscancer och magsäckscancer än det globala genomsnittet. Sedan några decennier ökar förekomsten av körtelbildande cancer i övergången mellan matstrupe och magsäck dramatiskt i västvärlden, medan magsäckscancer minskar över hela världen. Paradoxalt nog är infektion med bakterien Helicobacter pylori en skyddande faktor mot matstrupscancer samtidigt som infektionen kraftigt ökar risken för magsäckscancer, och man tror att en stor del av förklaringen till den ändrade förekomsten av de olika cancerformerna är den minskade förekomsten av helicobacter-infektioner i världen. Mekanismen bakom detta orsakssamband tros vara den förändring i syramiljö som bakterien skapar. I magsäcken är syrahalten normalt hög och i matstrupen är den låg. Ibland kan syra spilla över från magsäcken upp i matstrupen och sänka syrahalten där. Som svar på detta ändras matstrupens ytskikt, blir metaplastiskt, för att bättre tåla syran. Den metaplastiska vävnaden kan via förändringar i cellernas arvsmassa leda vidare till cancer. Infektion med Helicobacter pylori ger en lägre syrahalt i magsäcken, vilket kan leda till metaplasi och cancerutveckling i magsäcken samtidigt som det minskar risken för att syra ska komma upp i matstrupen.

Behandlingen av de båda cancerformerna har traditionellt inneburit operation i de fall patientens allmäntillstånd tillåter det och tumörväxten inte hunnit bli alltför utbredd, och symptomlindrande cellgifter och strålning i övriga fall. På senare år har man sett förbättrade resultat när man givit cellgifter eller strålbehandling före operation. Det har även utvecklats enstaka nya sorters målinriktade läkemedel, som har en mer specifik verkningsmekanism än cellgifter och strålbehandling, vilket förlängt överlevnaden ytterligare något. Trots dessa framsteg är tyvärr sannolikheten att bli botad från matstrups- eller magsäckscancer låg, och överlevnaden ofta endast något år, dels för att sjukdomen ger diffusa symptom och ofta upptäcks i ett sent skede, dels eftersom cancerformerna i sig är aggressiva.

Därför finns ett stort behov av att hitta och utvärdera nya läkemedel och behandlingsstrategier där man optimerar nyttan av, och minimerar biverkningarna av, de läkemedel som står till buds. En förutsättning för detta är studier av tumörerna för att bättre kunna förstå vilka ämnen som skiljer tumörceller från normala celler och vilka som driver tumören framåt. Identifiering av sådana ämnen skapar förutsättning för att förutsäga prognos och för att påverka ämnena så att tumörens aggressivitet kan bromsas.

I detta avhandlingsarbete har tumörmaterial och klinisk information från två grupper av patienter med matstrups- och magsäckscancer studerats, en grupp som opererats direkt (patientgrupp 1), och en som opererats efter cellgiftsbehandling (patientgrupp 2). Av vävnadsmaterialet har så kallade tissue microarrays, TMAs, framställts. Dessa är en form av vävnadschips där små vävnadsprover sätts samman i ett paraffinblock. På tunna snitt av detta paraffinblock kan sedan analyser av proteinuttryck i vävnadsbitarna göras. Sådan analys görs med immunhistokemi, det vill säga inmärkning av proteiner i vävnad med hjälp av antikroppar mot det protein som undersöks, som sedan synliggörs med färg och kan utvärderas i ett mikroskop. Tekniken möjliggör analys av ett stort antal vävnadsprover och proteinuttrycket kan sedan kopplas till patientöverlevnad. En nackdel med metoden är att eftersom vävnadsbitarna är små finns en risk att proteinuttrycket man ser inte speglar uttrycket i hela tumören. Även andra tekniker har använts i avhandlingsarbetet, såsom Western blot för att bestämma mängd av ett visst protein i en vävnad, siRNA transfektion för att blockera uttrycket av en viss gen och qPCR för att kontrollera att genuttrycket blivit blockerat. Dessa tekniker har främst använts för att utvärdera att de antikroppar som använts i TMA-analyserna fungerat korrekt. Därutöver har vi använt oss av pyrosekvensering för leta efter mutationer, dvs förändringar i cellernas arvsmassa.

De proteiner som undersökts är bl a SATB1, SATB2, KRAS, EGFR/HER1, HER2 och HER3. SATB1 och SATB2 är båda proteiner som kan binda till cellers DNA och påverka vilka gener som uttrycks och vilka proteiner som tillverkas. Tidigare studier har visat att SATB1 uttrycks i aggressiva tumörer, men det har funnits tveksamheter kring ifall de SATB1-antikroppar som använts eventuellt även binder in till SATB2, vilket skulle göra att man inte kan dra några säkra slutsatser av de resultat man får. SATB1-uttryck i matstrupscancer har inte tidigare studerats. I det första delarbetet visar vi att den SATB1-antikropp vi använder är väl fungerande, och vi visar att SATB1-uttryck är en markör för sämre överlevnad i vår undersökta patientgrupp 1.

EGFR/HER1, HER2 och HER3 är en grupp membranbundna receptorer, det vill säga proteiner som sitter i cellens yta, dit en ligand, ett slags signalprotein, kan binda in till delen som sticker ut utanför ytmembranet som en nyckel i ett nyckelhål. Ligandbinding gör att receptorn ändrar form, vilket sätter igång en kedja av signaler inom cellen och påverkar celldelning, cellmognad och överlevnad. Ökad aktivering av någon av dessa receptorer har tidigare visats ge en mer aggressiv tumör i flera cancerformer, men någon säker sådan koppling har inte setts i matstrups- och magsäckscancer. Det har utvecklats läkemedel mot EGFR-receptorn, som visat sig förbättra prognosen för patienter med lungcancer som överuttrycker EGFR, och mot HER2-receptorn, som visat sig förbättra prognosen för patienter med bröst- och magsäckscancer som överuttrycker HER2-receptorn. Det faktum att läkemedel mot EGFR-receptorn inte haft någon effekt

för patienter med magsäckscancer och att något läkemedel mot HER3-receptorn inte finns speglar å andra sidan de stora kunskapsluckor som fortfarande återstår. Det har heller inte i någon större utsträckning undersökts hur uttrycket av dessa proteiner påverkas av cellgiftsbehandling, vilket är en angelägen fråga med tanke på att cellgifter sedan ett par år är standardbehandling för patienter med matstrups- och magsäckscancer och att dessa patienter även kan komma att få läkemedel mot HER2-receptorn. Ytterligare ett område som är bristfälligt klarlagt är huruvida uttrycket av proteinerna skiljer sig åt mellan primärtumör och dottertumör, och ifall detta påverkar patientens prognos.

I det andra delarbetet har vi undersökt uttrycket av HER2 i normalvävnad, metaplastisk vävnad, primärtumörer och dottertumörer i patientgrupp 1. Vi såg att uttrycket förändrades från primärtumör till dottertumör i 13% av fallen, och att denna förändring av uttryck var kopplad till en sämre prognos. HER2-överuttryck i sig hade ingen koppling till prognos.

I det tredje delarbetet visar vi att den HER3 antikropp vi använder är väl fungerande, och vi undersökte uttryck av EGFR/HER1 samt HER3 i samma vävnader som i delarbete 2. Dessutom undersökte vi förekomsten av EGFR- och KRAS-mutationer. Vi såg en koppling mellan högt EGFR-uttryck och sämre överlevnad. Ett högt HER3-uttryck gav en trend mot, men ingen signifikant, överlevnadsfördel. Förändring av EGFR- respektive HER3-uttryck hade ingen påverkan på överlevnaden. Endast ett fåtal (4%) KRAS-mutationer sågs, och inga tumörer var EGFR-muterade.

I det fjärde delarbetet undersökte vi uttryck av EGFR, HER2 och HER3 i den andra patientgruppen, de som fått cellgiftsbehandling före operation. Vi jämförde uttrycket av de undersökta proteinerna i de små vävnadsbiopsier som tagits före behandling och operation, för att fastställa diagnos, med uttrycket i de behandlade operationspreparaten. Vi jämförde även uttrycket i primärtumör med det i dottertumörer. Ett förändrat uttryck före och efter behandling sågs i 5, 6 respektive 20% av tumörerna, för de respektive proteinerna. Dessa förändringar hade ingen påverkan på överlevnaden för patienterna. Även i denna andra patientgrupp sågs en antytt förlängd överlevnad för patienter med högt HER3-uttryck, men ingen överlevnadskoppling sågs för de andra proteinerna. Vad gäller förändrat uttryck mellan primärtumör och dottertumör sågs även i denna patientgrupp en antydan till försämrad överlevnad för de patienter som hade förändrat uttryck, men antalet patienter var i denna grupp för litet för att några säkra slutsatser ska kunna dras.

Vi undersökte även hur tumörerna svarat på behandlingen genom att jämföra histopatologiskt utseende, dvs sjukliga förändringar i vävnaden, efter behandling med prognos. Vi såg en tydlig koppling mellan histopatologiskt svar på behandling och överlevnad, där patienter med tumörer som påverkats kraftigt av

behandlingen hade en bättre överlevnad än de med tumörer som påverkats i mindre utsträckning.

Sammanfattningsvis ger våra resultat ytterligare bevis för att SATB1-uttryck ger en sämre prognos och att framtida försök att blockera SATB1-uttryck skulle kunna ge en överlevnadsvinst för de drabbade patienterna. Våra studier belyser även nya aspekter av HER-uttryck, koppling till prognos och av förändringar i uttryck under en tumörs framväxt, spridning och svar på behandling, vilket kan komma att påverka diagnostiskt tillvägagångssätt och behandlingsstategi.

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Charlotta Hedner, MD, studied medicine at the University of Gothenburg. She has now returned to her hometown Lund, where she is doing her pathology residency at the University hospital of Skåne. She is married and has two children.

The main aim of this thesis was to study the prognostic and predictive value of selected biomarkers in upper gastrointestinal cancer in order to identify novel, clinically relevant subgroups of the disease.

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