TECHNISCHE UNIVERSITÄT MÜNCHEN

Institut für Allgemeine Pathologie
und Pathologische Anatomie
der Technischen Universität München
(Klinikum rechts der Isar)

Molecular and phenotypical characterization of gastric cancer cell lines and validation of potential resistance factors to trastuzumab therapy

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Vollständiger Abdruck der von der Fakultät für Medizin
der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften
(Dr. rer. nat.)

genehmigten Dissertation.

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Die Dissertation wurde am 08.11.2018 bei der Technischen Universität München eingereicht und durch die Fakultät für Medizin am 21.03.2019 angenommen.

Contents

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADCC	antibody dependent cellular cytotoxicity
APS	ammonium persulfate
ASR	age-standardised rate
bp	base pair
BSA	bovine serum albumin
Ca^{2+}	Calcium
CIN	chromosomal instability
CISH	chromogenic in situ hybridization chromogen
CO_2	carbon dioxide
DAG	diacylglycerol
DAPI	4´,6-Diamidin-2´-phenylindol-dihydrochlorid
DGC	diffuse gastric cancer
DNA	deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
Dpi	dots per inch
ECD	extracellular domain
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
et al.	and others (*et alterii*)
FCS	fetal calf serum
FDA	Food & Drug Administration
FFPE	formalin-fixed paraffin-embedded
GDP	guanosine diphosphate
GTP	guanosine triphosphate
H_2O_2	hydrogen peroxide
HCl	hydrogen chloride
H&E	hematoxylin and eosin
HER	human epidermal growth factor receptor

HRP	horseradish peroxidase
ICD	intracellular domain
IGC	intestinal gastric cancer
IgG	immunoglobulin G
IHC	immunohistochemistry
IP3	inositol trisphosphate
L-CAM	liver cell adhesion molecule
MeOH	methanol
MET	hepatocyte growth factor receptor (HGFR)
Mg^{2+}	magnesium
MP	milk powder
MSI	microsatellite instability
NaCl	sodium chloride
NaF	sodium fluoride
NaOH	sodium hydroxide
$Na_2P_2O_7$	tetrasodium pyrophosphate
Na_3VO_4	sodium orthovanadate
NRG1	neuregulin 1
NSCLC	non-small cell lung cancer
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
Pen/Strep	penicillin/streptomycin
PBS	phosphate buffered saline
pEGFR	phosphorylated epidermal growth factor receptor
PMSF	phenylmethylsulfonphl fluoride
PVDF	polyvinylidene fluoride
rpm	rounds per minute
RNA	ribonucleic acid
RPMI	Roswell park memorial institute
RT	room temperature
RTK	receptor tyrosine kinase
S	serine
SDS	sodium dodecyl sulfate
T	threonine
TBE	tris/borate/EDTA
TBS	tris-buffered saline
TBS-T	tris-buffered saline with Tween

TEMED	Tetramethylethylenediamine
TKI	tyrosine kinase inhibitor
Tris	tris(hydroxymethyl)aminomethane
UCCL	University Cancer Center Leipzig
V	volume
v/v	volume per volume
VARIANZ	Vorhersage von Ansprechen oder Resistenz durch Identifizierung von Biomarkern beim Adenokarzinom des Magens und des gastroösophagealen Übergangs innerhalb einer nicht-interventionellen Studie und zielgerichteter Therapie mit Trastuzumab
Y	tyrosine

Physical value

%	percent
°C	degree centigrade
A	ampere
Da	dalton
g	gram
h	hour
l	liter
m	meter
min	minute
mol	mol
M	Molar [mol * l^{-1}]
MW	molecular weight
s	second
V	volt
W	watt

Physical quantities

M	mega
k	kilo
c	centi
m	milli
µ	micro
n	nano
p	pico

ABSTRACT

Gastric carcinoma is the fifth most common and the third leading cancer type in cancer-related deaths worldwide. HER2-positive gastric carcinomas are currently removed surgically and treated with a combination of HER2-targeted monoclonal antibody trastuzumab with chemotherapy.

The molecular mechanism and the direct effects on the gastric cancer signaling network of trastuzumab are only partially understood. In the present study, the effects of trastuzumab on gastric cancer cell lines were analyzed on proteomic and phenotypic levels, in order to identify predictive response and resistance factors. The response to trastuzumab was analyzed in gastric cancer cell lines, and potential biomarker candidates were investigated to predict response to trastuzumab therapy. A comprehensive panel of gastric cancer cell lines was screened for molecular and phenotypic response to trastuzumab treatment. Furthermore, HER2 expression was analyzed by immunohistochemistry (IHC), and *HER2* amplification was measured by chromogenic *in situ* hybridization (CISH). The classification of the gastric cell lines into responder and non-responder to trastuzumab treatment was based on proliferation data, as well as on the effects of trastuzumab on HER2 activation. Moreover, the possibility of further motility analyses was required. The effect of trastuzumab treatment varied across cell lines. Whilst NCI-N87 was identified as responder, cell lines MKN1 and MKN7 were clearly non-responder. Phenotypic response to trastuzumab of the cell lines MKN1, MKN7 and NCI-N87 was examined by invasion, motility and proliferation analysis. Furthermore, the molecular effects of trastuzumab on the receptor tyrosine kinase network and the downstream acting intracellular kinases were investigated. A more detailed molecular analysis using proteome profiling was performed to compare the effects of trastuzumab and the pan-HER kinase inhibitor afatinib. To evaluate these effects, data were analyzed using mixed-effect-models and clustering. The effects of trastuzumab differed across cell lines, depending on the presence of activated HER2. No effect of trastuzumab on MKN1 and MKN7 was observed in any of the aforementioned analyses. Trastuzumab monotherapy had a small effect on the trastuzumab responder cell line NCI-N87, as observed in molecular and phenotypic assays. These small effects did not affect transduction to the intracellular kinase network, as observed in proteome profile analysis results. Afatinib alone or in combination with trastuzumab influenced the HER kinases in all cell lines and effects were transduced to the intracellular kinase network. HER2-shedding and AXL were analyzed as potential resistance factors to trastuzumab. However, HER2-shedding was not affected by trastuzumab treatment in cell lines MKN1, MKN7 and NCI-N87, as observed in western blot and ELISA analyses. Molecular and phenotypic analyses also failed to demonstrate any association between AXL expression and trastuzumab treatment response in MKN7 cells.

ZUSAMMENFASSUNG

Das Magenkarzinom stellt weltweit die fünfthäufigste Krebserkrankung und die dritthäufigste krebsbedingte Todesursache dar. Gegenwärtig sieht die Standardtherapie von HER2-positiven Magenkarzinomen eine operative Entfernung des Tumors vor. Zusätzlich werden entsprechende Patienten mit einer Kombinationstherapie bestehend aus dem monoklonalen HER2-gerichteten Antikörper Trastuzumab und einer Chemotherapie behandelt.

Der molekulare Mechanismus des Antikörpers Trastuzumab und dessen direkten Auswirkungen auf das Signalnetzwerk sind bisher nicht komplett geklärt. In der vorliegenden Arbeit wurden Magenkarzinom-Zelllinien unter Therapie mit Trastuzumab auf proteomischer und phänotypischer Ebene analysiert, um Prädiktions- und Resistenzfaktoren zu identifizieren. Biomarker, die ein Ansprechen auf die Trastuzumab-Therapie vorhersagen könnten wurden bestimmt und untersucht. Verschiedenste Magenkarzinom-Zelllinien wurden molekular und phänotypisch auf ihr Ansprechens auf Trastuzumab charakterisiert. Des Weiteren wurde in diesen Zelllinien die HER2-Expression durch Immunhistochemie und die *HER2*-Amplifikation durch Chromogen-in-situ-Hybridisierung bestimmt. Eine Einteilung der Zelllinien in Responder und Non-Responder Zelllinien wurde anhand der Reaktion auf die Trastuzumab-Behandlung in der Proliferationsanalyse und der HER2-Aktivierung vorgenommen. Des Weiteren musste die Möglichkeit einer weiterführenden Motilitätsanalyse gegeben sein.

Die Wirkung der Behandlung mit Trastuzumab variierte über die Zelllinien hinweg. Die Zelllinie NCI-N87 wurde als Responder-Zelllinie, wohingegen die Ziellinien MKN1 und MKN7 als Non-Responder-Zelllinien gegenüber einer Trastuzumab-Behandlung identifiziert wurden. Die phänotypische Charakterisierung der Zelllinien MKN1, MKN7 und NCI-N87 erfolgte mittels Invasions-, Motilitäts- und Proliferationsanalyse. Molekulare Effekte von Trastuzumab auf Rezeptortyrosinkinasen und intrazelluläre Kinasen wurden ermittelt. Des Weiteren wurde durch *Proteome profiling* eine detaillierte molekulare Analyse der Wirkungen von Trastuzumab und dem pan-HER-Kinase-Inhibitor Afatinib erreicht. Die Bewertung der erhaltenen Effekte ermöglichte ein *Mixed-effect-model* und eine Clusteranalyse.

Als entscheidend für die Wirkung von Trastuzumab in den Zelllinien stellte sich das Vorhandensein von aktiviertem HER2 heraus. In den Trastuzumab-Non-Responder-Zelllinien MKN1 und MKN7 wurden keine Trastuzumab-bedingten Effekte beobachtet. In molekularen und phänotypischen Analysen zeigten sich unter Trastuzumab-Therapie geringe Effekte für die Responder-Zelllinie NCI-N87. Diese Effekte übertrugen sich nicht auf das intrazelluläre Kinasennetzwerk der Zelllinie, wie die Analyse durch P*roteome profiling* zeigte. Die Kombination

aus Trastuzumab mit Afatinib, und eine alleinige Afatinib-Therapie zeigte starke Effekte auf die HER-Kinasen und das intrazelluläre Kinasennetzwerk in allen Zelllinien.
Als potenzielle Resistenzfaktoren gegenüber Trastuzumab-Therapie wurden das HER2-*Shedding* und die Rezeptortyrosinkinase AXL untersucht. Durch molekulare Analysen konnte keine Assoziation des Ansprechens auf Trastuzumab und HER2-*Shedding* in allen drei Magen-Karzinomzelllinien festgestellt werden. Molekulare und phänotypische Analysen konnten keinen Zusammenhang zwischen der Expression von AXL und dem Ansprechen auf Trastuzumab in der Zelllinie MKN7 nachweisen.

1 INTRODUCTION

1.1 Oncology

Tumors can arise in every organ of the human body, due to uncontrolled cell proliferation and are distinguished into benign and malign tumors. Benign tumors are well differentiated and characterized by slow tumor growth. Benign tumor cells show epithelial structures, normal cell-cell adhesion and do not infiltrate other tissues. Infiltration of surrounding tissue by tumor cells leads to the definition of the tumor as malign. The cells of a malign tumor are undifferentiated and show multiple and enlarged cell nuclei. An important characteristic of malignant tumor cells is their invasive capabilitiy and ability to form metastases. Benign tumors are classified by their origin into adenomas (gland tissue), fibromas (connective tissue), lipomas (adipose tissue) and myomas (muscle tissue). Malign tumors are classified into carcinomas, lymphomas/leukemias and sarcomas. Carcinomas develop from epithelial cells. Lymphomas develop from lymphatic tissue and leukemia cells of the hematopoietic system. Sarcomas develop from connective tissue and can be further divided into angiosarcomas (from blood vessels), liposarcomas (from adipose tissue) and myosarcomas (from muscle tissue) (Weinberg 2014).

1.1.1 Tumorigenesis

The transformation of a healthy cell into a tumor cell is caused by several individual events (Kinzler and Vogelstein 1996, Vogelstein and Kinzler 1993, Weinberg 2014). Three main stages can be distinguished: initiation, latency/promotion, and progression. Initiation occurs through mutations in genes important for cell division or cycle such as tumor suppressors (Boyd and Barrett 1990, Pitot 1993). The second hit hypothesis states that a tumor suppressor gene mutation event on only one allele is not sufficient to transform cells, but a second allele must be also altered, in order to cause a homozygote mutation (Knudson 1979). During latency/promotion, cell division is stimulated by growth factors. Non-genotoxic carcinogens or hormones act as growth stimuli, causing proliferating cells to pass on mutations to their offspring. During progression, further mutations in tumor suppressor genes and increased conversion of proto-oncogenes into oncogenes occur. Malignant tumor cells accumulate, dedifferentiate, become invasive and metastasize (Boyd and Barrett 1990, Pitot 1993). Hanahan and Weinberg summarized six features that are responsible for the progression of malignant cells including independence from growth signals, insensitivity to growth inhibition, bypassing apoptosis, unlimited cell division and the ability to induce

angiogenesis, invasion and metastasis (Weinberg 2014). New insights in the progression of carcinomas led to the definition of four additional hallmarks: reprogramming of the cellular energy metabolism to support cell growth, ability to evade immune system attacks, genome instability and mutations, and inflammatory status of premalignant lesions, which exploits the immune system to promote tumor progression (Hanahan and Weinberg 2011).

1.1.2 Metastasis

The progression of carcinomas is a multi-step process in which primary tumors can form secondary tumor metastases. In the subsequent process of the metastatic cascade, the ability of tumor cells to be motile allows them to invade, disseminate, and metastasize in distant, pre-metastatic niches of distant and surrounding tissue (Alberts, Schäfer, and Häcker 2011). In order to form metastases, cells of the primary tumor must be able to transmigrate the extracellular matrix (ECM) by proteolysis (Kassis et al. 2001). The epithelial-mesenchymal transition enables neoplastic tumor cells to detach from the primary tumor site. After intravasation, tumor cells are transported through the bloodstream or lymphatic vessels to other organs, where the malignant cells can form a pre-metastatic niche (Friedl and Wolf 2003). Following adhesion to the epithelium of an organ, extravasation takes place and the cells invade the target organ to build micrometastases. When the micrometastases reach a size of approx. 1 mm^3, the supply of oxygen and nutrients is no longer sufficient, thus, neoangiogenesis follows (Alberts, Schäfer, and Häcker 2011, Gupta and Massague 2006, Hanahan and Folkman 1996).

1.1.2.1 Migration

Migration is the result of sequential, dynamic processes in cells. Different signaling pathways, adhesion dynamics and actin-myosin contractions regulate the change in cell morphology and motility (Burridge and Chrzanowska-Wodnicka 1996, Cramer 1999, Lamalice, Le Boeuf, and Huot 2007). Crucial steps are cell polarization with directional stretching of the membrane and formation of lamellipodia, followed by protuberance of a cell portion by a leading edge and stabilization by the adhesion to the ECM (Abercrombie, Dunn, and Heath 1977, Friedl and Brocker 2000). Actin polymerization and assembly into filaments is the initial step of the extension of the leading edge and adherence to the ECM. Involved structures are invadopodia, filopodia, lamellipodia and pseudopodia (Adams 2001). During cell adhesion, integrins or receptors, acting as cell adhesion molecules, cluster and form a contact surface between the cell membrane and the substrate (Hynes 2002). The integrin clustering and local assembly of integrin types is dynamic, allowing

the cell to slide along the substrate surface. The integrin repertoire on the surface is specific for each cell type and depends on the substrate (Friedl and Brocker 2000). *In vitro* substrates are for example, fibronectin, vitronectin and collagen. Depending on the substrate, cells form specific integrin dimer complexes of α and β chains (Cukierman et al. 2001, Leavesley et al. 1992, Rabinovitz and Mercurio 1997). Subsequent contraction of the cell body and the leading edge enables the cell to glide along the substrate (Adams 2001). The motility of cells is influenced by intrinsic and extrinsic factors. Intrinsically, the movement is influenced by the actin cytoskeleton, whereas extrinsic factors, such as concentration gradients, regulate the directionality of cell movement. Chemotaxis describes cell movement along a concentration gradient. In a concentration equilibrium of chemotactically active substances, random chemokinetic movement occurs (Wells et al. 2002).

1.1.2.2 Invasion

Invasiveness is mandatory for cells to migrate actively through the cellular blood system. A subpopulation of tumor cells must be able to recognize and degrade the ECM (Wells 2000). Therefore, two distinct cellular mechanisms are necessary: enzymatic cleavage of the ECM components collagen, fibronectin, and proteoglycan, as well as a cell-induced physical rearrangement and reorientation of these components (Friedl and Wolf 2009, Wolf et al. 2007).

1.2 Gastric cancer

In 2018, gastric cancer was the fifth most common and third leading cancer responsible for cancer-related deaths worldwide (Bray et al. 2018).

Worldwide, almost one million patients are newly diagnosed with approximately 700,000 cases of death each year (Jackson et al. 2007). Especially in countries, such as China, Chile, Colombia, Japan and Korea high incidences and mortality rates of gastric cancer are recorded (Figure 1). In general, the gastric cancer rate is about twice as high in males as in females (Gonzalez, Sala, and Rokkas 2013). The five-year survival rate, which is often used to assess the malignancy of a tumor, is less than 25 % for gastric carcinoma worldwide (Duraes et al. 2014). In Germany, the five-year survival rate is about 80 % at stadium I, 60 % at stadium II, 30 % at stadium III and < 5 % at stadium IV (Ferlay et al. 2015). Most cases occur in patients above the age of 50, with rare cases before the age of 30 (Kelley and Duggan 2003).

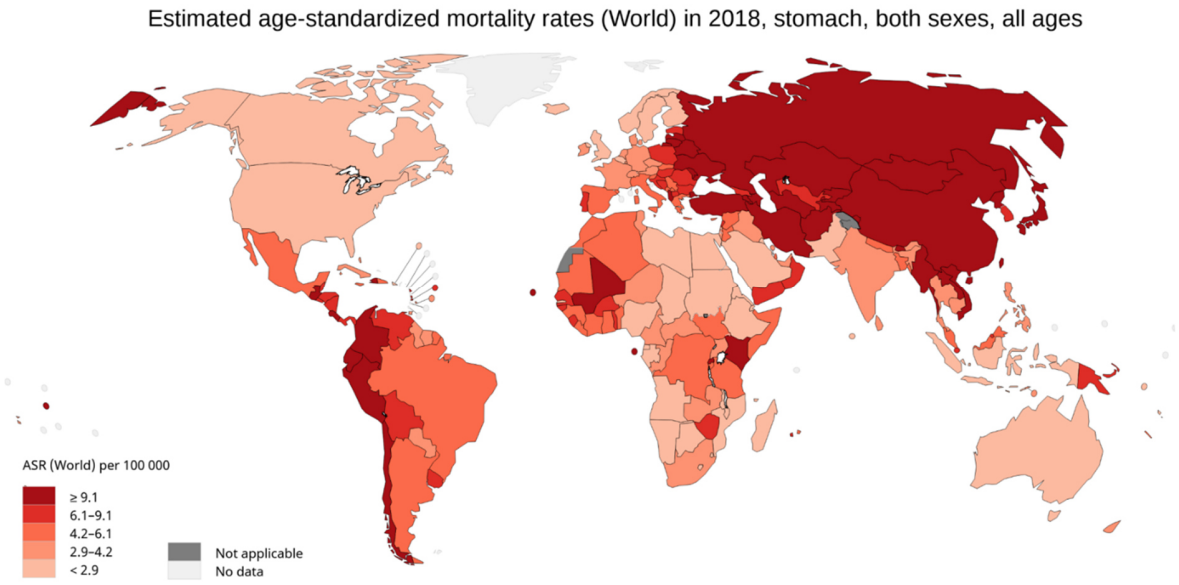


Figure 1: Mortality of gastric cancer worldwide in 2018.
Number of recorded deaths per 100,000 persons in 2018 in different countries worldwide. Color intensity correlates with the frequency of gastric cancer related death (Bray et al. 2018).

1.2.1 Anatomy of the stomach

The stomach is located in the left upper abdomen under the diaphragm. It is divided into five sections: entrance/cardia, fundus, body/corpus, antrum and pylorus. The cardia, esophagus and the stomach are lined with mucosa and submucosa. Through the cardia, food passes from the esophagus to the stomach. The food is stored in the fundus before it is mixed with gastric juice and enzymes by muscles of the corpus. Subsequently, the food is transported on to the antrum and pylorus. The muscles of the antrum and the pylorus further mince and mix the food porridge. The mucous membrane of the antrum produces hormones to control digestion (Schwegler and Lucius 2011).

1.2.2 Genetic, molecular and general risk factors

The development of gastric cancer has been associated with different environmental, infectious and host-related risk factors. Some examples are high age, low economic status, smoking and excessive alcohol consumption (Guggenheim and Shah 2013, Wang, Terry, and Yan 2009, Zali, Rezaei-Tavirani, and Azodi 2011). Dietary habits such as low vegetable and fruit consumption or frequent consumption of fat, meat, strongly salted, grilled, cured or smoked foods also increase the risk of gastric cancer (Piazuelo and Correa 2013, Tkachenko et al. 2007, Wadhwa et al. 2013). Moreover, studies concerning nitrite and nitrosamines revealed a positive correlation with gastric

cancer incidence (Jakszyn and Gonzalez 2006). Over decades a steady decrease in the incidence and mortality of gastric carcinoma was monitored in most countries (Munoz and Franceschi 1997). This decrease could be due to improvements in food preservation and storage (Roosendaal et al. 1997). Another group of risk factors is various pre-existing gastric diseases. For example, patients who suffer from chronic gastritis, atrophy, intestinal metaplasia or dysplasia have an increased risk to develop gastric cancer (Armstrong and Doll 1975).

A possible cause of gastritis or stomach ulcers is an infection with the bacterium *Helicobacter pylori* (*H. pylori*). Adenocarcinomas can arise in a systematic process called the Correa model, also known as Correa's cascade described by Correa et al. in 1975 (Correa et al. 1975). Following the identification of *H. pylori* in 1983, it was recognized that this process is initiated and sustained by the infection with this bacterium (Correa and Chen 1994, Warren and Marshall 1983). In 2008, 78 % of 989,000 gastric carcinoma patients had previously been infected with *H. pylori* (Herrero, Parsonnet, and Greenberg 2014). Overall *H. pylori* is estimated to be involved in the onset of 60 – 70 % of all gastric cancers (Gonzalez, Sala, and Rokkas 2013). The gram-negative bacterium colonizes the stomach and damages the gastric mucosa (Marshall and Warren 1984, Warren and Marshall 1983). The attachment of *H. pylori* to gastric epithelial cells leads to chronic inflammation and causes tissue damage (Wadhwa et al. 2013). Generally, the infection with *H. pylori* is acquired in childhood (Parkin 2006). An infection with *H. pylori* increases the likeliness to develop gastric cancer to 2 - 3 times over individuals who never suffered from this infection. However, not every patient with Helicobacter-induced gastritis or ulcer develops gastric cancer (Cancer Genome Atlas Research 2014, Wroblewski, Peek, and Wilson 2010, Yaghoobi, Bijarchi, and Narod 2010).

Genetic predisposition increases the risk for gastric cancer about 1.5 - 3.5 times if a first-degree family member already suffers from gastric cancer (Bakir et al. 2000, Yaghoobi, Bijarchi, and Narod 2010). Most alterations associated with gastric cancer are acquired (Sereno et al. 2011). Less than 15 % of all gastric cancer cases are due to familial clustering (Zanghieri et al. 1990), whereas < 5 % are linked to hereditary syndromes (Bornschein and Malfertheiner 2011, La Vecchia et al. 1992, Vogiatzi et al. 2007). The most frequent hereditary gastric cancer syndrome is hereditary diffuse gastric carcinoma (HDGC) (Guilford et al. 1999). HDGC is associated with a germline mutation in the *CDH1* gene, including deletion, frameshift mutation, splice-site mutation or missense mutations (Oliveira et al. 2013). Furthermore, sporadic polyps and syndromic polyps represent an increased risk of developing gastric carcinoma. These polyps are characteristic for patients suffering from the familial adenomatous polyposis (FAP) syndrome (Ushijima and Sasako 2004). The FAP syndrome represents an autosomal dominant disorder. It is caused by a germline mutation of the tumor suppressor gene adenomatous polyposis coli (*APC*) (Islam et al. 2012).

Other genetic risk factors include epigenetic changes and mutations in *BRCA2* (Thorlacius et al. 1996), *E-cadherin*, *ErbB3*, *KRAS*, *PIK3CA*, *PTEN*, *STK11* (Ushijima and Sasako 2004) and *TP53* (Cancer Genome Atlas Research 2014, Gonzalez, Sala, and Rokkas 2013, Okines et al. 2010, Varley et al. 1995). In addition, defects in the DNA repair system leading to microsatellite instability (MSI) are associated with gastric carcinoma. In MSI, defective DNA repair proteins cause a change in length of short repetitive sequences, for example in hereditary non-polyposis colorectal cancer (HNPCC) (Choi et al. 2014, Duraes et al. 2014). Most of the genetic alterations associated with gastric cancer are caused by chromosomal instability (CIN), functional single nucleotide polymorphisms, MSI or somatic gene mutations leading to dysregulated signaling pathways (Hanahan and Weinberg 2011).

1.2.3 Classification

The classification by Lauren is a histological classification of gastric carcinoma, in which tumors are classified into three types. The three types are intestinal gastric cancer (IGC) (54 % of all cases), diffuse gastric cancer (DGC) (32 % of all cases) and a mixed form of the two types (15 % of all cases) (Cancer Genome Atlas Research 2014, Hu et al. 2012, Lauren 1965, Polkowski et al. 1999). The IGC shows differentiated intestinal and gland structures that form mucins as well as tubular structures. The cohesive tumor cells are well differentiated with only few stromal components. This type grows locally limited, expansive into the stomach lumen comparable to a polyp and can be clearly distinguished from the surrounding healthy tissue. IGC is strongly associated with chronic inflammation and IGC appears more frequently in males and older age groups. In contrast, cells of the DGC are poorly differentiated and the proportion of stroma is significantly increased (Kelley and Duggan 2003, Lauren 1965). These poorly differentiated gastric cells form infiltrating cell aggregates, which can cause tissue stiffening (Lynch et al. 2005). The tumor grows diffuse and infiltrative, making it difficult to distinguish healthy from tumor tissue (Kakiuchi et al. 2014). The DGC is more common in women and in the younger demographic. Patients with intestinal gastric carcinoma have a better prognosis than those with a diffuse gastric carcinoma (Kelley and Duggan 2003, Kim, Sierke, and Koland 1994, Lauren 1965). A subset of DGC is the signet ring cell carcinoma, which evolves from glands. In addition to the two forms, a less common mixed tumor type exists, in which characteristics of both types can be found (Hu et al. 2012, Kelley and Duggan 2003, Lauren 1965).

An entirely new subdivision of gastric carcinoma was published in 2014 as a part of The Cancer Genome Atlas Project (TCGA). The aim of the project was to classify gastric carcinomas on a

molecular level and to identify dysregulated signaling pathways in the gastric cancer cell lines. A comprehensive genome-wide analysis of 295 primary gastric carcinomas resulted in the classification of four subtypes based on genetic criteria. The first subtype consists of Epstein-Barr virus positive tumors (EBV+, 9 %). Characteristics of this subtype are mutations in *PIK3CA*, a pronounced hypermethylation of the DNA and amplifications in *JAK2.* The second subtype is represented by tumors with MSI (21 %). Characteristic are an increased mutation rate in genes that are involved in oncogenic signaling pathways and hypermethylation in the MutL homolog 1 (*MLH1*) (Duraes et al. 2014, Queiros et al. 2015). Genomically stable (GS) tumors (20 %) form the third subtype. Histologically, GS tumors are diffuse and mutations in *CDH1* can be found. In addition, fusions occur between genes encoding RHO family GTPase activating proteins. The fourth subtype that is described includes chromosomally instable tumors (CIN, 50 %). Typical for CIN tumors are amplifications of receptor tyrosine kinases and mutations in *TP53* (Cancer Genome Atlas Research 2014). EBV+ and MSI tumors are suspected to have a better prognosis than the other gastric cancer types (Hu et al. 2012).

The World Health Organization offers an alternative system classifying gastric tumors into intestinal and diffuse types. According to their growth structure, tumors are subdivided into tubular, papillary and mucinous adenocarcinomas as well as into signet ring cell carcinomas (Piazuelo and Correa 2013). The TNM classification of Malignant Tumours classifies carcinomas into different stages according to their progression. The abbreviation T stands for the size of the primary tumor (TX, Tis, T0-T4). The presence of regional lymph node metastases is indicated by N (NX, N0-N3) and M stands for the presence of distant metastases (M0, M1) (Wittekind 2015).

The Borrmann classification is based on the growth properties of tumors. Type 1 tumors often show polyps. Tumors characterized by ulcerations are distinguished into type 2 tumors with sharp margins and type 3 tumors are defined by loss of well-defined margins. Non-ulcerated, diffuse and infiltrative tumors are referred to as type 4 tumors (Borrmann 1926). Further classifications divide tumors through gene amplifications. This classification results in five tumor subgroups: EGFR (7.7 %), FGFR2 (9.3 %), HER2 (7.2 %), MET (4.0 %) and KRAS (8.8 %) (Schulte, Ebert, and Härtel 2014). Thus, 35 % of the gastric cancer patients are predicted to respond to RTK / RAS-targeted therapy (Deng et al. 2012).

1.3 Human epidermal growth factor receptor family

The receptor tyrosine kinases of the HER family, which consists of EGFR, HER2, HER3 and HER4, are linked to human cancer pathogenesis (Arteaga and Engelman 2014). The four receptors are expressed in epithelial, mesenchymal and neuronal cells and their respective progenitors (Roskoski 2014). The HER receptors show a conserved structure. This structure consists of an extracellular ligand-binding domain composed of four glycosylated subdomains, a hydrophobic transmembrane region with a hydrophobic anchor sequence, a cytoplasmic tyrosine kinase domain and a carboxy-terminal region with multiple tyrosine residues (Figure 2) (Hudis 2007, Jorissen et al. 2003, Olayioye et al. 2000, Tao and Maruyama 2008). The extracellular domain, in contrast to the intracellular domain, is less highly conserved, allowing different ligands to bind to the receptors (Hynes and Lane 2005, Normanno et al. 2006).

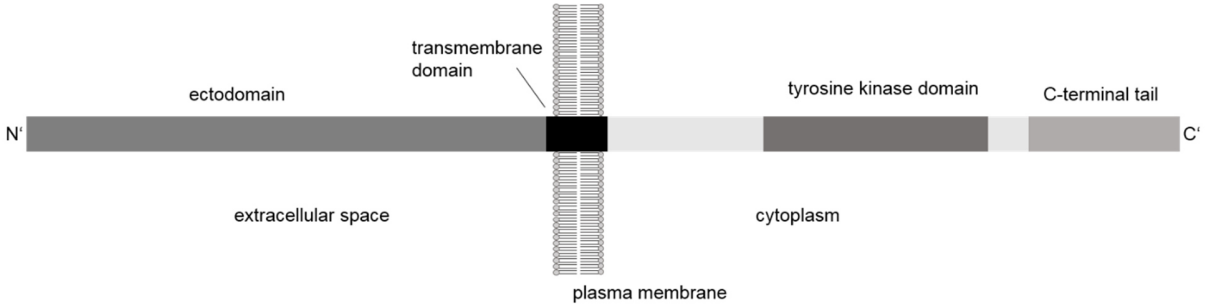


Figure 2: Conserved general structure of the HER family.
All members of the HER family show a conserved structure. The N-terminal extracellular ectodomain is responsible for ligand binding. The transmembrane domain is located in the plasmamembrane. The intracellular tyrosine kinase domain is responsible for signal transduction. The cytoplasmic end composes the C-terminal tail.

In the inactivated conformation of the receptor, the extracellular domain 2 binds to domain 4, blocking the dimerization arm sterically, thus allowing no interaction with other receptors (Figure 3). This auto-inhibits the inactive receptor. Ligand binding alters the orientation of domain 2, enabling dimerization (Normanno et al. 2006, Roskoski 2004). To activate EGFR, HER3 and HER4 ligand-binding is necessary. These ligands can act in an autocrine, paracrine or juxtacrine way (Harris et al. 1993, Singh and Harris 2005). The precursor proteins of the ligands in the cell membrane are processed and released by proteolysis (Massague and Pandiella 1993).

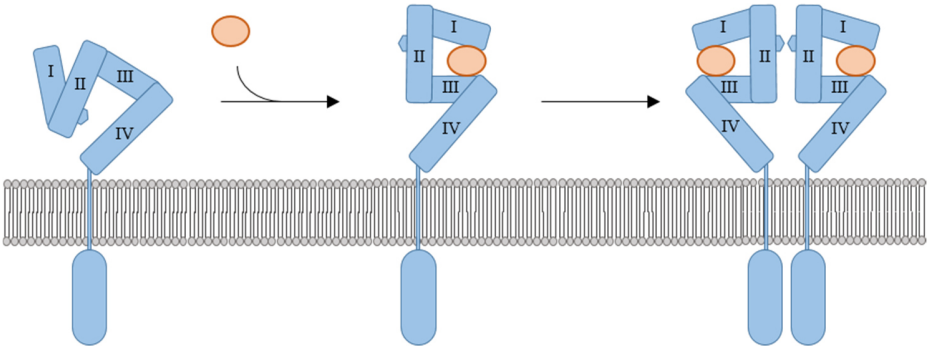


Figure 3: Ligand induced conformational changes of HER receptors
The inactive HER receptor is activated by ligand-binding and subsequent conformational changes. In the inactivated conformation of the receptor, the extracellular domain II binds to domain IV, blocking the dimerization arm sterically. This auto-inhibits the inactive receptor. Ligand-binding alters the orientation of domain II, enabling dimerization. Thus, the interaction with other receptors is possible.

Three different groups of ligands are known (Figure 4). The first group consisting of amphiregulin (AREG), epigen (EPG), EGF and TGF-α can activate EGFR. Furthermore, EGFR and HER4 are activated by β-cellulin (BTC), epiregulin (EPR) and HB-EGF, representing the second group of ligands. The third group of ligands are NRG1 and NRG2 binding to HER3 and NRG3 and NRG4 binding to HER4 (Normanno et al. 2006, Schneider and Wolf 2009). While EGF, HB-EGF and TGF-α are high affinity ligands, AREG is a low affinity ligand (Sanders et al. 2013). The respective ligands bind the extracellular domain of the monomeric receptor, resulting in a conformational change of the receptor. Receptor homodimers and heterodimers with other family members can be formed leading to trans-autophosphorylation of the cytoplasmic tyrosine kinase domains. The activated tyrosine kinases serve as binding sites for additional signaling molecules and adapter proteins, triggering various further signaling pathways (Arteaga and Engelman 2014, Hudis 2007, Hynes and Lane 2005, Roskoski 2014, Yarden and Sliwkowski 2001). The activated downstream protein kinase cascades are associated with the regulation of cell growth and survival, adhesion, migration and differentiation (Yarden 2001b).

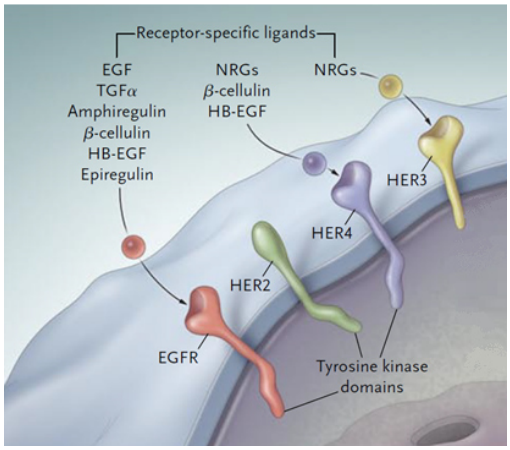


Figure 4: HER ligands
The EGF receptor is activated by AREG, β-cellulin, EGF, EPG, EPR, HB-EGF and TGF-α. The ligands β-cellulin and HB-EGF furthermore activate HER4. The ligand group consisting of NRGs activate HER4 and HER3. HER2 has no ligand-binding domain ((Ciardiello and Tortora 2008) modified).

1.3.1 Human epidermal growth factor receptor 1

The human epidermal growth receptor 1 (EGFR) is also known as HER1 and was the first member of the HER family to be described (Carpenter 1987, Cohen, Carpenter, and King 1980, Downward et al. 1984, Yarden and Sliwkowski 2001). EGFR is glycosylated and has a molecular mass of 185 kDa. The coding gene comprises of 26 exons and is located on chromosome 7p12. Crystallographic analysis of the extracellular region of EGFR had shown that subdomains 1, 2 and 3 form a ligand-binding pocket (Garrett et al. 2002, Ogiso et al. 2002). The seven different ligands known for EGFR are AREG, BTC, EGF, EPG, EPR, HB-EGF and TGF-α (Normanno et al. 2006, Sanders et al. 2013). EGF leads to the degradation of EGFR, whereas AREG, EREG and TGF-α induce receptor recycling (Roepstorff et al. 2008, Roepstorff et al. 2009). After the binding of a ligand, EGFR homo- or heterodimerizes with other receptors via subdomain 2 (Li et al. 2005, Schlessinger 2000). EGFR-heterodimers can be formed with HER2 and HER3, but not with HER4 (Wells 1999). The EGFR-HER2 heterodimer is more stable than other dimers, because HER2 lowers ligand dissociation and inhibits recycling through endocytosis (Yarden 2001b). EGFR is involved in different signaling pathways. Among these pathways are the regulation of cell proliferation, motility, apoptosis, invasion and angiogenesis (Harari, Allen, and Bonner 2007, Zandi et al. 2007). Null mutations of *EGFR* in mice lead to multiple organ failure and are lethal (Greulich et al. 2005, Ji et al. 2006, Miettinen et al. 1995, Politi et al. 2006). The stringent regulation of the EGFR activation system can be overcome by certain mechanisms. These include the autocrine production of ligands, heterodimerization and interaction with other membrane-associated structures, impaired inactivation mechanisms, and mutations in the *EGFR* gene (Cappuzzo et al.

2005, Frederick et al. 2000, Nishikawa et al. 1994, Pao et al. 2005, Zandi et al. 2007). Another described mechanism is the overexpression of EGFR, which is observed in about 27 % of all gastric-carcinomas and can be caused by *EGFR* gene amplification or an increased *EGFR* promoter activity (Kim, Lee, et al. 2008, Lemoine et al. 1991, Yokoyama et al. 2006). High EGFR expression in patients is often associated with advanced tumor stage, shorter survival, resistance to standard therapies and poor prognosis (Arteaga 2002, Capdevila et al. 2009, Holbro, Civenni, and Hynes 2003). The current therapeutic strategies targeting EGFR include monoclonal antibodies directed against the extracellular receptor domain and small molecule tyrosine kinase inhibitors (TKIs) targeting the intracellular tyrosine kinase domain. Despite several TKIs, two phase 3 trials REAL3 (Waddell et al. 2013) and EXPAND (Lordick et al. 2013) failed to demonstrate an improvement in overall survival with an EGFR directed therapy in patients with advanced gastric or esophageal cancer (Yang, Raufi, and Klempner 2014).

1.3.2 Human epidermal growth factor receptor 2

The gene for the human epidermal growth factor receptor 2 (HER2) is a proto-oncogene, located on chromosome 17. It encodes the 185 kDa transmembrane receptor HER2 (Qian et al. 1994, Yarden and Sliwkowski 2001). HER2 is expressed in various tissues, including breast, heart, kidney and the gastrointestinal tract. HER2 alterations can be found in roughly 6 - 34 % of all gastric cancer patients, with the highest rates in the intestinal type (David et al. 1992, Samson and Lockhart 2017, Yang, Raufi, and Klempner 2014). HER2 is overexpressed in about 5 - 25 % of all gastric cancers (Fuse et al. 2016, Kim, Kim, Im, et al. 2008). In contrast to the other HER receptors HER2 has no ligand-binding domain. The crystal structure of HER2 shows a constitutively active form that is similar to ligand-activated EGFR (Cho et al. 2003, Citri, Skaria, and Yarden 2003, Yarden and Pines 2012). Thus, HER2 kinase activity can be induced in the absence of a ligand. HER2 is able to dimerize with itself or other HER family members after they have been activated by ligand binding (Brennan et al. 2000, Hynes and Lane 2005, Marchini et al. 2013, Qian et al. 1994, Wolf-Yadlin et al. 2006). The preferred binding partner of HER2 is HER3, causing a strong signal transduction (Baselga and Swain 2009, Holbro, Civenni, and Hynes 2003, Hynes and Lane 2005, Olayioye et al. 2000). HER2-overexpression may result in homodimers that induce constitutive activation of the most important signaling pathways downstream of HER2, RAS-ERK and PI3K-AKT (Arteaga and Engelman 2014, Dittrich et al. 2014, Roskoski 2014, Yokoyama et al. 2006). HER2-positive breast cancer is associated with poor overall survival and relapse after therapy compared to HER2-negative breast cancer (Boku 2014). There is also evidence for poor

prognosis in HER2 overexpressing gastric carcinoma (Garcia et al. 2003, Gravalos and Jimeno 2008, Junior, Neto, and Forones 2016, Qiu et al. 2014). However, Janjigian et al. published longer survival of patients with HER2-positive gastric carcinoma than patients with HER2-negative gastric tumors (Janjigian et al. 2012). In addition to the regular form of HER2, different truncated forms of the receptor exist. The truncation of HER2 by proteolytic cleavage results in an extracellular (HER2 ECD) and intracellular domain of HER2 (HER2 ICD) which is constitutively activated (Normanno et al. 2006). The lack of the extracellular domain makes HER2 ICD resistant to antibody therapy, but kinase inhibitors continue to be effective (Arteaga and Engelman 2014).

1.3.3 Human epidermal growth factor receptor 3

HER3 is the third receptor of the HER family with its coding gene located on chromosome 12 (Yarden and Sliwkowski 2001). HER3 is expressed in several tissues such as the respiratory, urinary or gastrointestinal tract (Kol et al. 2014). HER3 positivity is ranging from 25 % to 30 % in gastric cancer patients (Yokoyama et al. 2006). The ligands NRG1 and NRG2 can bind to the extracellular domain of HER3, resulting in a conformational change and heterodimerization with other HER receptors (Garner et al. 2013, Normanno et al. 2006, Schneider and Wolf 2009, Yuan et al. 2017). HER3 can only form heterodimers due to a very weak kinase activity (~ 1/1000 of the other HER receptors) (Baselga and Swain 2009, Shi et al. 2010). The preferred dimerization partners are EGFR and HER2 with HER3 serving as an effective phosphotyrosine scaffold for the activation of downstream signaling (Graus-Porta et al. 1997, Kol et al. 2014, Shi et al. 2010). Furthermore, a heterotetramer by which HER2 phosphorylates HER3 and an adjacent HER2 homodimer phosphorylating HER2 is possible (Tzahar et al. 1996). Various studies indicate that HER3 overexpression is associated with poor survival observed in many cancer entities such as brain tumors (Takikita et al. 2011), breast cancer (Giltnane et al. 2009, Shimoyama 2014) and gastric carcinoma (Hayashi et al. 2008, He et al. 2015, Yun et al. 2015). Increased HER3 activity may bypass the inhibition of other HER receptors, which is evident in the sustained activity of the PI3K-AKT signaling pathway (Dey et al. 2015, Jura et al. 2009, Kim, Sierke, and Koland 1994, Sergina et al. 2007, Soltoff et al. 1994).

1.3.4 Downstream signaling

The three best studied downstream signaling pathways of the HER receptors are the Ras-ERK (Ras extracellular signal-regulated kinases), the PI3K-PKB/AKT (phosphatidylinositol 3'-kinase-protein kinase B) and the PLCγ-PKC (phosphoinositide-phospholipase C γ-protein kinase C) pathway (Figure 5). The PI3K-PKB/AKT pathway mainly controls cell survival, whereas the Ras-ERK pathway is mainly responsible for proliferation (Nyati et al. 2006).

1.3.4.1 Ras-ERK pathway

In the Ras-ERK signaling pathway, adapter proteins are recruited after phosphorylation of the cytoplasmic tyrosine residues of the receptors. Two different ways to activate the Ras-ERK signaling pathways are known: either directly via SH2-mediated recruitment of growth factor receptor-bound protein 2 (GRB2) or indirectly via PTB-mediated binding of the SHC (Src homology and collagen protein) adapter.

The GRB2-associated guanine nucleotide exchange factor Sos (Son of sevenless) activates Ras via the exchange of guanosine diphosphate (GDP) with guanosine triphosphate (GTP). Consequently, Ras undergoes a conformational change allowing the binding and activation of the Raf kinase. The phosphorylation and consequent activation of serine residues of MEK1/2 and tyrosine and threonine residues of ERK1/2 finally leads to the activation of various proteins, such as the ERK-activated protein kinase and the ribosomal p70-S6 kinase. Finally, transcription factors and genes responsible for cell differentiation, growth, motility, migration, proliferation and survival are influenced (Dittrich et al. 2014, Marais et al. 1995, Marmor, Skaria, and Yarden 2004, Nishinaka and Yabe-Nishimura 2001, Nyati et al. 2006, Schlessinger and Bar-Sagi 1994).

1.3.4.2 PI3K-PBK/AKT pathway

The HER receptors activate PI3K. Through dimerization of PI3K, the subunit p85 is recruited to the cytoplasmic phosphorylated tyrosine kinases of the HER receptors. This allows PI3K to phosphorylate phosphatidylinositol-4,5-bishoshpat (PIP2) and induce the formation of phosphatidylinositol-3,4,5-triphosphat (PIP3). PIP3 triggers a cascade of phosphorylation until the activation of AKT, which has cytoplasmic and nuclear functions. Active AKT causes the inhibition of the proliferation-inhibitor GSK-3β, of pro-apoptotic BAD (BCL2 antagonist of cell death), procaspase 9, Forkhead Box Protein (FOXO) transcription factors and the inhibition of the protein synthesis inhibitor TSC2. AKT promotes cell proliferation, invasion and growth (Dillon, White, and Muller 2007, Dittrich et al. 2014, Hennessy et al. 2005, Marmor, Skaria, and Yarden 2004, Nyati et al. 2006). AKT also mediates activation of mTOR, which promotes protein translation (Marmor,

Skaria, and Yarden 2004, Soltoff and Cantley 1996). Active AKT also induces angiogenesis through the activation of HIF-1α (Weinberg 2014).

1.3.4.3 PLCγ-PKC pathway

Activated EGFR and HER2 can bind SH2 recruiting PLCγ to the cell membrane. PLCγ hydrolyzes PIP2 into the secondary transporters diacylglycerol (DAG) and inositol trisphosphate (IP3). Binding of IP3 to the endoplasmic reticulum causes an intracellular calcium increase and consequently activates different calcium-dependent enzymes. DAG serves as a cofactor for PKC, which leads to cell cycle progression, transformation, differentiation and apoptosis (Marmor, Skaria, and Yarden 2004, Schlessinger 2000).

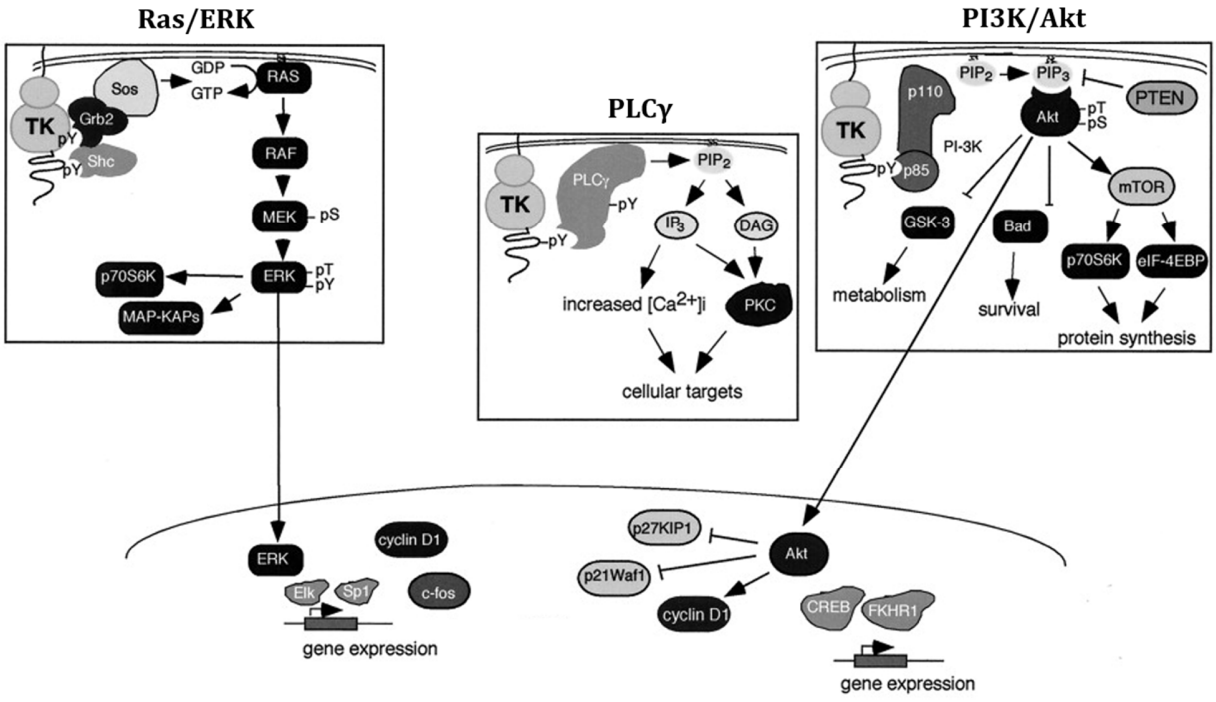


Figure 5: Downstream signaling pathways of HER receptors
The activation of HER receptors results in the activation of tyrosine kinase activity and the phosphorylation of specific tyrosine residues. Signaling effectors are recruited to the activated receptors and induce various signaling pathways as the Ras-ERK, the PI3K-PKB/AKT and the PLCγ-PKC pathway. The PI3K-PKB/AKT pathway mainly controls cell survival, whereas the Ras-ERK pathway is mainly responsible for proliferation ((Marmor, Skaria, and Yarden 2004) modified).

1.4 Therapy options

Depending on the progression, gastric carcinomas are treated only surgically or surgically with adjuvant drug therapy. In early stage gastric carcinoma (with altered mucosa without ulcer), gastric mucosa is removed by endoscopy or partial gastrectomy (Krams et al. 2013). In advanced gastric carcinomas the common therapeutic option is a gastric resection (at least to the smooth muscle

of the stomach). Furthermore, lymph nodes are removed and perioperative chemotherapy is given (Chon et al. 2017). Most chemotherapy treatments are based on the combination of at least two cytostatica such as epirubicin, cisplatin, capecitabine and 5-fluorouracil (Lordick et al. 2014, Wagner et al. 2006). These drugs damage cancer cells by causing defects in cell cycle control or in DNA repair mechanisms in order to inhibit cell division. However, toxic effects are observed in many healthy cells (Alberts, Schäfer, and Häcker 2011, Kang et al. 2009, Van Cutsem et al. 2006, Vanhoefer et al. 2000).

New therapy options were developed in the last years. Therapeutic antibodies bind to ligands or receptors whereas small molecules are able to enter cancer cells. Due to occurring resistance mechanisms, investigations for precise biomarkers are necessary (Groenendijk and Bernards 2014). Appropriate agents appear to be active against EGFR, FGFR2, HER2, MET, VEGF/VEGFR or against members of the PI3K-PKB/AKT signaling pathway (Schulte, Ebert, and Härtel 2014). Tumors that express different HER receptors are associated with a more aggressive phenotype and a worse prognosis (Normanno et al. 2006). Since February 2015, ramucirumab, a monoclonal human anti-VEGFR2 antibody with an anti-angiogenic effect, has been approved in Germany as a second-line treatment of gastric carcinoma. Studies with antibodies directed against HGF or cMET had to be terminated prematurely due to lack of efficacy. Nintedanib, a multi-tyrosine kinase inhibitor capable of inhibiting FGFR, PDGFR and VEGFR signaling could provide therapeutic benefit (Lordick et al. 2015).

1.4.1 Afatinib

The small molecule inhibitor afatinib is an irreversible ATP competitor that binds covalently to the cysteine residues of the HER receptors (Arteaga and Engelman 2014). Although afatinib can inhibit all HER receptors, the spectrum and severity of side effects are similar to other inhibitors (Eskens et al. 2008). Afatinib is metabolized only slightly and no circulating metabolite has been identified so far (Li et al. 2008). In 2013, the European Medicines Agency (EMA) and the Food & Drug Administration (FDA) approved afatinib for the treatment of non-small cell lung cancer (NSCLC) with *EGFR* mutation. The inhibitory concentrations of afatinib are 0.5 nM for wild-type EGFR and 14 nM for HER2 (Solca et al. 2012). The maximum tolerated dose of afatinib in HER2-overexpressing gastric cancer is currently investigated in combination with trastuzumab treatment (Boehringer 2017). Furthermore, the combination of afatinib with chemotherapy is evaluated in three phase 2 studies. One study analyzes the combination of afatinib with cisplatin and 5-fluorouracil as a first-line therapy in advanced gastric cancer (Hellenic 2017). In EGFR-positive

gastric cancer the combination of afatinib and paclitaxel is presently being evaluated as a second-line therapy (Yonsei 2017). In refractory esophagogastric cancer another phase 2 study investigates the combination of afatinib with paclitaxel and trastuzumab (MSKCC 2017).

1.4.2 Lapatinib

Lapatinib is a dual tyrosine kinase inhibitor that inhibits EGFR and all forms of HER2 (Kim, Kim, Im, et al. 2008, Shimoyama 2014). Lapatinib reversibly binds to the ATP binding domain of the kinases, preventing signal transduction of the Ras-ERK pathway (Konecny et al. 2006, Spector et al. 2005). For breast cancer, the inhibitor is approved for treatment of patients expressing high levels of EGFR and HER2 (Cameron et al. 2008, Geyer et al. 2006, Scaltriti et al. 2007). The analysis of lapatinib in phase 2 and 3 studies did not improve the overall survival of patients with gastric carcinoma (Lorenzen et al. 2015, Satoh et al. 2014, Woo, Cohen, and Grim 2015).

1.4.3 Cetuximab

Cetuximab is a monoclonal chimeric antibody directed against EGFR (Figure 6) (Adams and Weiner 2005, Capdevila et al. 2009, Vincenzi et al. 2010, Yewale et al. 2013). The antibody sterically prevents the conformational change of the closed EGFR monomer into the upright, dimerization-active form (Li et al. 2005). This allows cetuximab to inhibit the ligand-dependent activation of EGFR homo- and heterodimers (Capdevila et al. 2009, Fan et al. 1994, Patel et al. 2009). Cetuximab binds to the subdomain 3 of the extracellular domain, thereby blocking the ligand-binding sites (Hubbard 2005). Furthermore cetuximab binds EGFR with a 50-fold higher affinity than EGF or TGF-α, thus ligand-induced EGFR phosphorylation, downstream signaling and EGFR internalization is inhibited (Li et al. 2005, Patel et al. 2009). In addition, the cell cycle of the cells remains in G0/G1 arrest, apoptosis is induced and antibody dependent cellular cytotoxicity (ADCC) is initiated (Kimura et al. 2007, Yewale et al. 2013). In 2004, the FDA and EMA approved cetuximab for EGFR-positive advanced colorectal cancer and in 2006 for recurrent or metastatic squamous-cell head and neck carcinoma. The EMA approval restricts cetuximab to *KRAS* and *NRAS* wild types, because activating mutations in *KRAS* or *NRAS* lead to constantly activated EGFR downstream signaling (Arteaga and Engelman 2014, Meriggi et al. 2014, Van Cutsem et al. 2011, Vermorken et al. 2008, Yewale et al. 2013). In gastric cancer the frequency of *KRAS* mutations is low (Kim et al. 2003). The open-labeled phase 3 study EXPAND in advanced gastric cancer investigating the efficiency of cetuximab with capecitabine and cisplatin, compared to chemotherapy alone failed to show additional benefit of cetuximab. However, the molecular

status of patients has not been considered (Lordick et al. 2013, Okines, Cunningham, and Chau 2011).

1.4.4 Pertuzumab

Pertuzumab is a monoclonal humanized antibody that binds to domain 2 of the HER2 receptor and sterically blocks the dimerization domain (Figure 6) (Agus et al. 2002, Franklin et al. 2004). Thereby, pertuzumab inhibits homo- and heterodimerization, both in the presence and in the absence of ligands (Agus et al. 2002, Baselga 2002, Franklin et al. 2004). Synergistic effects with trastuzumab were shown in preclinical and clinical studies (Garner et al. 2013, Gianni et al. 2012, Scheuer et al. 2009). Since 2012, pertuzumab is approved by the FDA in combination with trastuzumab and docetaxel for the treatment of HER2-positive metastatic breast cancer (Moya-Horno and Cortes 2015). In HER2-positive, advanced gastric cancer and gastroesophageal junction cancer the effect of pertuzumab in combination with trastuzumab and chemotherapy was evaluated in a phase 2a trial (Kang et al. 2014). This trial was the basis for the phase 3 JACOB study, which failed. In this study, the combination of pertuzumab with trastuzumab, fluoropyrimidine, and cisplatin was investigated in HER2-positive metastatic gastric and gastroesophageal junction cancer (NCT01774786) (ClinicalTrials 2018, Kang et al. 2014).

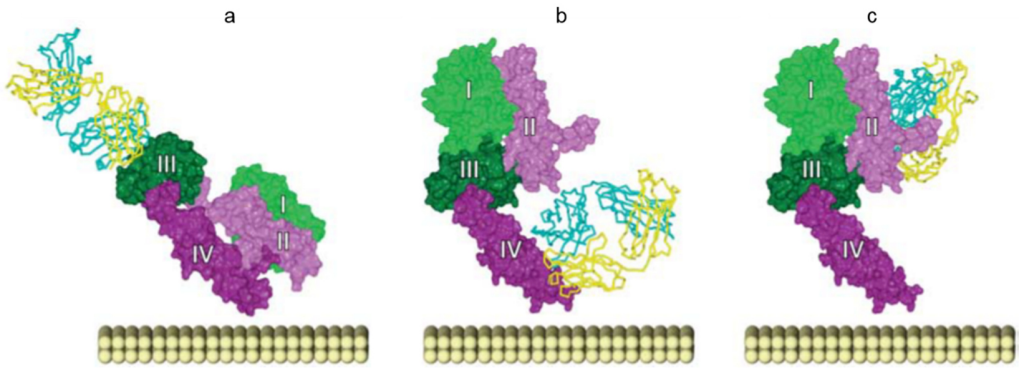


Figure 6: Structure of cetuximab, trastuzumab and pertuzumab bound to the HER receptors.
The antibody cetuximab (a) binds to the ectodomain III of EGFR. Trastuzumab (b) and pertuzumab (c) bind the HER receptor HER2. Ectodomain IV is bound by trastuzumab, whereas pertuzumab binds to the ectodomain II (Hubbard 2005).

1.4.5 Trastuzumab

The monoclonal humanized antibody trastuzumab specifically binds HER2 (Figure 6). Trastuzumab consists of two hypervariable regions with antigen-specific binding sites for the subdomain 4 of the juxtamembrane region of HER2. Subdomain 4 serves as one of the two dimerization sites of HER2. The backbone of the antibody represents a human IgG antibody with a constant Fc-region (Gajria and Chandarlapaty 2011, Hubbard 2005, Okines and Cunningham

2012). In 1998, the antibody was approved by the FDA, and in 2000 by the EMA, for the treatment of metastatic breast cancer with HER2 overexpression. In early and metastatic HER2-positive breast cancer patients an advantage in overall survival was shown, which makes trastuzumab today`s standard of care in breast cancer therapy (Smith et al. 2007). In 2010, the approval was extended from breast cancer to the treatment of advanced and metastatic HER2-positive gastric cancer in combination with 5-fluoropyrimidine or capecitabine plus cisplatin. The approval was based on the results of the Trastuzumab for Gastric Cancer study (ToGA) in 2009 (Bang et al. 2010, Leto et al. 2015, Lordick et al. 2015). In this international phase 3 study, the use of trastuzumab in addition to standard chemotherapy in patients with advanced and inoperable HER2-positive gastric cancer was examined. In combination with chemotherapeutics, the antibody increased the mean overall survival of HER2-positive gastric cancer patients from 11.1 months with chemotherapy alone to 13.8 months with chemotherapy plus trastuzumab (Bang et al. 2010). This combination therapy is now considered as standard first-line therapy for HER2-positive gastric carcinoma. Previous testing for HER2 by immunohistochemistry (IHC) and/or fluorescence/chromogenic in situ hybridization (FISH/CISH) is necessary (Lordick et al. 2014). FISH detects the amplification of HER2 with a fluorescent-labeled probe. IHC staining is used to determine the expression of HER2 using a HER2-specific antibody (Alberts, Schäfer, and Häcker 2011). The therapeutic response to gastric carcinoma was increased by 12 % with trastuzumab. Nevertheless, the overall response rate to trastuzumab in combination with chemotherapy is below 50 % for all gastric cancer patients (Shimoyama 2014). In some patients, tumor recurrence occurs within 7 months (Bang et al. 2010, ClinicalTrials 2018, Lordick et al. 2014, Samson and Lockhart 2017, Satoh et al. 2014). Several possible mechanisms of action are discussed. Trastuzumab is able to sterically block ligand-independent receptor dimerization, thus HER2-mediated signaling is inhibited (Baselga 2002, Gajria and Chandarlapaty 2011, Lane et al. 2001, Valabrega, Montemurro, and Aglietta 2007). Contrary, trastuzumab is unable to block ligand-induced HER2-containing heterodimers (Agus et al. 2002). The antibody increases the internalization and endocytic degradation of HER2 and shows antiangiogenic effects (Hudis 2007, Izumi et al. 2002, Klos et al. 2003, Ohta and Fukuda 2004, Okines and Cunningham 2012, Sliwkowski et al. 1999, Yarden 2001a). Furthermore, trastuzumab inhibits the proteolytic cleavage of HER2, preventing the shedding of HER2 to the membrane-bound constitutively active HER2 ICD (Molina et al. 2001). In addition to direct molecular effects, trastuzumab shows immunogenic effects. Effector cells of the immune system are recruited via the Fc fragment. Macrophages and natural killer cells build the ADCC leading to cell death of the tumor cells (Clynes et al. 2000, Cuello et al. 2001, Ghosh et al. 2011, Sliwkowski et al. 1999).

1.4.5.1 Resistance mechanisms to trastuzumab treatment

Different resistance mechanisms to trastuzumab therapy are known (Niederst and Engelman 2013, Tse et al. 2012). Only subgroups of tumor cells are responsive to trastuzumab, and even in therapy-sensitive groups, resistance can occur over time (Nahta and Esteva 2006, Samson and Lockhart 2017, Zheng et al. 2014). Several mutations in *HER2* are reported, which might be associated with resistance (Kwong and Hung 1998, Lee et al. 2006, Mitra et al. 2009). Various receptor tyrosine kinases compensate the inhibition of signaling pathways by trastuzumab via alternative signaling pathways such as EGFR (Wheeler et al. 2008), HER3 (Berns et al. 2007, Garrett et al. 2011, Junttila et al. 2009, Montero et al. 2008, Motoyama, Hynes, and Lane 2002, Sergina et al. 2007, Wheeler et al. 2008), IGF-1R (Lu et al. 2001), MET (Minuti et al. 2012, Shattuck et al. 2008, Turke et al. 2010) and other proteins (Arteaga and Engelman 2014, De Luca et al. 2012, Deng, Jing, and Meng 2013, Eto et al. 2015, Khoury et al. 2005, Nahta and Esteva 2006, Narayan et al. 2009, Organ and Tsao 2011, Price-Schiavi et al. 2002, Zheng et al. 2014). HER ligands seem to promote resistance against trastuzumab, too (Rexer and Arteaga 2013, Ritter et al. 2007, Wang et al. 2008, Yotsumoto et al. 2010). Despite the binding of trastuzumab to HER2, the downstream signaling pathway can be further maintained by genetic alterations such as mutations of the PI3K catalytic subunit (*PIK3CA*) or low PTEN expression (Berns et al. 2007, Kang, Bader, and Vogt 2005, Karakas, Bachman, and Park 2006, Majewski et al. 2015, Nagata et al. 2004, Rexer and Arteaga 2013, Velho et al. 2005, Vu, Sliwkowski, and Claret 2014, Yakes et al. 2002). Furthermore, the overexpression of proteases such as ADAM17 can result in an increased HER2-shedding, resulting in the constitutively active truncated form of the HER2 receptor (Miller, Sullivan, and Lauffenburger 2017).

1.4.5.1.1 HER2-shedding

HER2-shedding is a known resistance mechanism in breast cancer and is reported to be related to the responses to trastuzumab (Colomer et al. 2000, Leitzel et al. 1995, Scaltriti et al. 2007). The full-length receptor HER2 is cleaved by matrix metalloproteases. The extracellular domain of HER2 (HER2 ECD) is released and can be detected in cell culture media (Lin and Clinton 1991, Pupa et al. 1993, Zabrecky et al. 1991). In patient serums, high levels of HER2 ECD correlate with a poor prognosis (Colomer et al. 2000, Esteva et al. 2002, Kostler et al. 2004, Leitzel et al. 1995, Yamauchi et al. 1997). *In vitro* HER2-targeted antibodies were shown to bind circulating HER2 ECD competitive to the binding of the full-length HER2 (Zabrecky et al. 1991). Furthermore, proteolytic cleavage also generates a membrane-associated HER2 ICD form and other fragments with a functional kinase activity (Anido et al. 2006, Christianson et al. 1998). These fragments of

the HER2 receptor lack the extracellular trastuzumab-binding domain (Christianson et al. 1998, Molina et al. 2002). High levels of HER2 ICD were reported to be associated with trastuzumab resistance or lower the response rate to trastuzumab in breast cancer. Additional or alternative strategies targeting HER2 are required in these patients (Scaltriti et al. 2007).

1.4.5.1.2 AXL

The *AXL* gene was discovered in chronic myeloid leukemia in 1988 and characterized in 1991 (Gay, Balaji, and Byers 2017, Liu, Hjelle, and Bishop 1988, O'Bryan et al. 1991). AXL is expressed in the endothelium of the capillaries, marrow stroma, myeloid cells, vascular smooth muscle cells, tumor cells and tumor vasculature (Neubauer et al. 1994, O'Donnell et al. 1999). The transmembrane receptor tyrosine kinase (RTK) belongs to the TAM family and is activated by binding of growth arrest-specific protein 6 (Gas6) (Varnum et al. 1995). When AXL is overexpressed, it can be activated ligand-independently by homodimerization (Brown et al. 2016). Homo- and heterodimerization with other receptors leads to trans-autophosphorylation of the tyrosine residues of the kinase domain and the effector molecules p85 and GRB2 are recruited to activate the downstream signaling pathways PI3K-AKT and Ras-ERK (Meyer et al. 2013, Scaltriti, Elkabets, and Baselga 2016, Vouri et al. 2016). AXL affects various cellular processes in development, growth and tumor progression, modulating proliferation, invasiveness, migration, epithelial-mesenchymal transition, angiogenesis and immunomodulation (Gay, Balaji, and Byers 2017, Korshunov 2012, Meyer et al. 2013, Ou et al. 2011, Paccez et al. 2013, Uribe et al. 2017, Zhang et al. 2008). Loss of function leads to chronic inflammation, which can cause autoimmunity (Rothlin et al. 2007). Overexpression of AXL has been reported in carcinomas of various entities including breast, esophagus, intestine, kidneys, lung and ovaries, correlating with a strongly negative prognosis (Brown et al. 2016, Gjerdrum et al. 2010). Correlations between AXL overexpression and therapy resistance have been reported for gastrointestinal stromal tumors towards imatinib (Mahadevan et al. 2007) and towards lapatinib and trastuzumab in HER2-positive breast cancer cell lines (Liu et al. 2009).

1.5 The SYS-Stomach consortium

This doctoral thesis is part of the BMBF-funded SYS-Stomach project (grant #01ZX1310). The SYS-Stomach consortium aims to identify response and resistance factors to EGFR and HER2 targeted therapy with cetuximab or trastuzumab in gastric carcinoma, using a systems medicine approach. Systems medicine is a research field that combines knowledge from life sciences with computer sciences to understand complex physiological and pathological processes in their

entirety. Central aims of the consortium are the identification of biomarkers and knowledge management, multilevel analysis of gastric cancer data, agent-based models of tumors for the definition of adjuvant therapy methods, in situ proteome analysis of gastric cancer and the clinical validation of the predictive response- and resistance factors in gastric carcinoma. This is realized by partners Prof. Birgit Luber (Institute of Pathology, Technical University Munich), Dr. Dieter Maier (Biomax Informatics AG), Prof. Fabian Theis, Dr. Jan Hasenauer and Prof. Axel Walch (Helmholtz Zentrum München Neuherberg), Prof. Michael Meyer-Hermann (Helmholtz Center for Infection Research in Braunschweig) and Prof. Florian Lordick (University Cancer Center Leipzig). The research goal of our subproject is the characterization of gastric cancer cell lines in response to treatment with trastuzumab. The effects of the treatment are determined on phenotypic and molecular level. Based on the obtained results, mathematical models are created: probabilistic models of basic signaling networks coupled to the cellular phenotypes and agent-based cellular behavior models. Potential biomarkers predicted by these models and biostatistical methods are then validated in cell culture. These candidates are then further analyzed on tumor collectives.

2 MATERIALS & METHODS

2.1 Materials

2.1.1 Cell lines

For the SYS-Stomach project, a panel of 12 gastric cancer cell lines was analyzed. Most of these cell lines were ordered from professional providers.

AGS, #89090402	European Collection of Cell Cultures (ECACC)/Sigma-Aldrich, Steinheim
GSU, #RCB2278	Cell Bank, Riken BioResource Center Koyadai, Japan
H-111-TC, #RCB1884	Cell Bank, Riken BioResource Center Koyadai, Japan
HGC-27, #RCB0500	Cell Bank, Riken BioResource Center Koyadai, Japan
Hs746T, #HTB-135™	American Type Culture Collection (ATCC)/LGC Standards GmbH, Wesel
HSC-45	C. Seidl, Klinikum rechts der Isar, München
KATO-III, #86093004	ECACC/Sigma-Aldrich, Steinheim
LMSU, #RCB1062	Cell Bank, Riken BioResource Center Koyadai, Japan
MKN1, #RCB1003	Cell Bank, Riken BioResource Center Koyadai, Japan
MKN7, #RCB0999	Cell Bank, Riken BioResource Center Koyadai, Japan
MKN45	Prof. Dr. M. Ebert, Klinikum rechts der Isar, München
NCI-N87, #CRL-5822™	ATCC/LGC Standards GmbH, Wesel

The cell line AGS originates from a diffuse adenocarcinoma of a 54-year-old Caucasian female. The patient did not receive any prior therapies (Datasheet AGS). Mutations in *KRAS* Exon 2 (c.35G>A, p.G12D), *PIK3CA* (c.1357G>A, p.E453K) and *CDH1* Exon 12 (c.1733_1734insC; p.G579fs*9) are reported (Bamford et al. 2004). GSU was established from a metastasis of an ascites of a diffuse gastric carcinoma from a 37-year-old Japanese male (Miyamoto et al. 2018). Few is known about the cell line H-111-TC, established from an epithelial-like intestinal gastric

carcinoma (Miyamoto et al. 2018). The cell line HGC-27 was derived from a lymph node. The tumor showed a poor differentiation and was isolated from a Japanese with unspecified sex (Akagi and Kimoto 1976). HSC-45 was derived from a ascites metastasis of a signet ring cell gastric adenocarcinoma from a 28-year-old female (Yanagihara et al. 1993). The histology of the primary tumor of the cell line Hs746T is unknown. It originates from a metastasis in the left leg muscle of a gastric adenocarcinoma from a 74-year-old Caucasian male (Smith 1979). The cell line KATO-III was established from a 55-year-old male patient with down syndrome. The metastasis of a pleural effusion was isolated of a signet ring cell gastric adenocarcinoma (Sekiguchi, Sakakibara, and Fujii 1978, Smith 1979, Yanagihara et al. 1991). The cell line LMSU was derived from a metastasis in a lymph node from a 27-year-old Japanese female (Datasheet LMSU). MKN1 was derived from a lymph node as metastatic site of a gastric adenosquamous carcinoma. The tumor of a 74-year-old Japanese male showed poor differentiation (Motoyama, Hojo, and Watanabe 1986). Mutations in *FBXW7* (c.1393C>T, p.R465C), *PIK3CA* (c.1633G>A, p.E545K) and *TP53* (c.428T>C, p.V143A, p.R210H) are reported (Bamford et al. 2004). The cell line MKN7 was derived from a lymph node metastasis of a moderately differentiated intestinal gastric tubular adenocarcinoma (Yokozaki 2000). For the primary tumor of the 39-year-old Japanese male mutations in *EGFR* (p.I923J) and *TP53* (p.278S) are reported (Miyamoto et al. 2018, Motoyama, Hojo, and Watanabe 1986). The cell line MKN45 was derived from a metastasis in the liver of a 62 year-old female. The differentiation of the diffuse tumor was reported to be poor (Motoyama, Hojo, and Watanabe 1986). From a liver metastasis of a well differentiated intestinal gastric cancer, the cell line NCI-N87 was isolated (Miyamoto et al. 2018). The primary tumor of the male American patient (age unknown) was well differentiated (Park et al. 1990). Mutations in *HER2* (p.F425L, p.D417D, p.S413L, p.L436V, p.T900Pb, p.R898Gb, p.Q902Kb, p.R897Gb, p.Q902Rb) and *TP53* (p.R248Q, p.R155Q) are reported (Arienti et al. 2016, Bamford et al. 2004).

All cell lines were grown and microscopically analyzed for morphology (Figure 7). Different size and shape were observed across the different gastric cancer cell lines. AGS cells have a compact shape, similar to HSC-45 cells. The cell lines GSU and KATO-III are morphologically related, showing small round-shaped cells with hardly visible lamello- or filopodia. H-111-TC and NCI-N87 cells grow in clusters. Compared to the other cell lines H-111-TC and NCI-N87 cells are very small. HGC-27, Hs746T and MKN1 cells are strongly attached, with visible filopodia. LMSU cells are more elongated with directed filopodia. In contrast, MKN7 cells have a compact shape with filopodia in all directions. MKN45 cells are small and round-shaped, growing on top of each other.

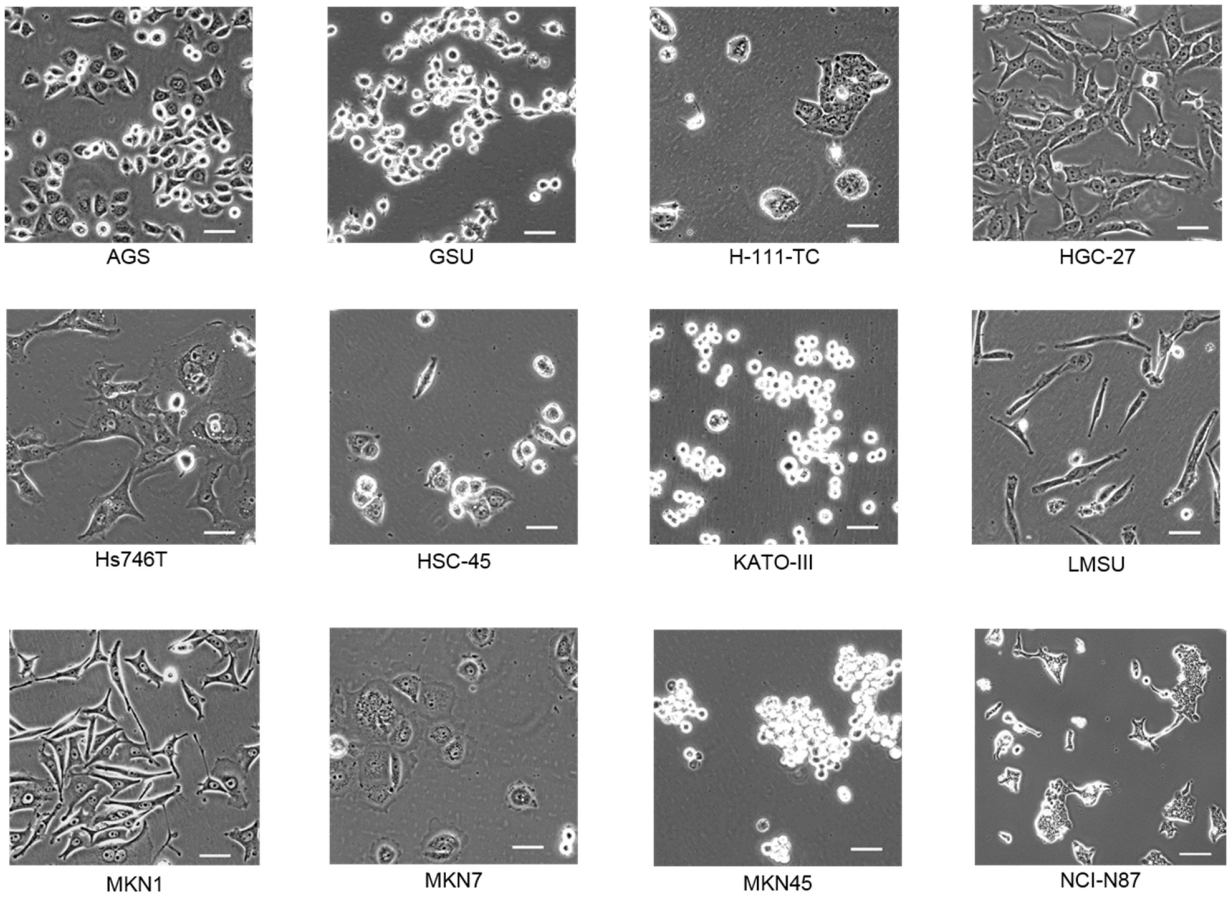


Figure 7: Pictures of all cell lines of the gastric cancer cell line panel.
For all cell lines, pictures were taken in a cell culture flask under regular culture conditions. Pictures were recorded with a phase contrast microscope with a 10 x resolution. The size bars represents 50 µm. Different morphological characteristics of the cell lines are recognizable.

2.1.2 Media and Solutions for cell culture

DMEM+GlutaMAX-I 500ml, #31966-021	ThermoFisher Scientific, Darmstadt
Dimethylsulfoxid (DMSO), #276855	Sigma-Aldrich, Steinheim
Dulbecco´s PBS with Ca^{2+}/ Mg^{2+}, #H15-002	PAA, Pasching, Österreich
Dulbecco's PBS without Mg^{2+}/Ca^{2+}, #L1825	Biochrom, Berlin
L-Glutamine (200 mM) 100ml, #25030-081	ThermoFisher Scientific, Darmstadt
Minimum Essential Medium Eagle, 500ml, #M4655-500ML	Sigma-Aldrich, Steinheim
Nutrient mixture F-10 Ham, 500ml, #N6908-500ML	Sigma-Aldrich, Steinheim
Opti-MEM I Reduced Serum Medium 500 mL, #31985070	ThermoFisher Scientific
Opti-MEM I Reduced Serum Medium, no phenol red, #11058-021	Life Technologies GmbH, Darmstadt

Penicillin/Streptomycin 100ml, 10000 U/ml (Penicillin 5.000 IU/ml, Streptomycin 5.000 µg/ml), #15140-122	ThermoFisher Scientific, Darmstadt
RPMI 1640 Medium (500ml), #11875093	ThermoFisher Scientific, Darmstadt
Sera Plus special processed fetal bovine serum (FBS), 500ml, #P30-3702	PAN Biotech, Aidenbach
Trypan blue 0,4% sterile-filtered, #T8154-20ML	Sigma-Aldrich, Steinheim
Trypsin-EDTA (0.05 %), phenol red, #25300054	ThermoFisher Scientific, Darmstadt
Versene solution 100ml, #15040066	ThermoFisher Scientific, Darmstadt

2.1.3 Antibodies

Anti-α-tubulin, mouse monoclonal clone DM1A, #T9026-100µl	Sigma-Aldrich, Steinheim
Anti-β-actin, mouse monoclonal clone AC-15, #A1978-100µl	Sigma-Aldrich, Steinheim
Anti-ErbB2 [CB11], mouse monoclonal, #ab8054	Abcam, Cambridge
Anti-Human c-erbB2, rabbit polyclonal, #A0485292	Dako, Hamburg
Anti-mouse IgG HRP-linked whole antibody from Sheep 100µl, #GENA931-100µl	Sigmal-Aldrich, Steinheim
Anti-rabbit IgG, HRP-linked, #7074P2	Cell Signaling/New England Biolabs (NEB), Frankfurt a.M.
AXL C89E7, rabbit monoclonal, #8661S	Cell Signaling/NEB, Frankfurt a.M.
EGF Receptor EGFR antibody, rabbit polyclonal, #2232S	Cell Signaling/NEB, Frankfurt a.M.
ErbB2 Antibody e2-4001 + 3B5, mouse monoclonal, #MA5-14057	ThermoFisher Scientific, Schwerte
HER2/ErbB2 29D8, rabbit monoclonal, #2165S	Cell Signaling/NEB, Frankfurt a.M.
HER3/c-erbB-3 Ab-2, rabbit monoclonal, #MS-201-P1	ThermoFisher Scientific, Schwerte
HER3/ErbB3 1B2, rabbit monoclonal, #4754S	Cell Signaling/NEB, Frankfurt a.M.
Human IgG1 Kappa-LE/AF, Isotype Control, #SBA-0151K-14	SouthernBiotech/Biozol, Eching
MET 25H2, mouse monoclonal, #3127S	Cell Signaling/NEB, Frankfurt a.M.
Phospho-Akt Ser473, rabbit polyclonal, #9271S	Cell Signaling/NEB, Frankfurt a.M.
Phospho-Akt Ser473 D9E XP, rabbit monoclonal, #4060S	Cell Signaling/NEB, Frankfurt a.M.

Phospho-AXL Tyr702 D12B2, rabbit monoclonal, #5724S	Cell Signaling/NEB, Frankfurt a.M.
Phospho-EGFR Tyr1068, rabbit polyclonal, #44-788G	ThermoFisher Scientific, Schwerte
Phospho-HER2/ErbB2 Tyr1221/1222 Antibody, rabbit polyclonal, #2249S	Cell Signaling/NEB, Frankfurt a.M.
Phospho-HER2/ErbB2 Tyr1248, rabbit polyclonal, #2247S	Cell Signaling/NEB, Frankfurt a.M.
Phospho-HER3/ErbB3 Tyr1222 50C2, rabbit monoclonal, #4784S	Cell Signaling/NEB, Frankfurt a.M.
Phospho-HER3/ErbB3 Tyr1289 21D3, rabbit monoclonal, #4791S	Cell Signaling/NEB, Frankfurt a.M.
Phospho-MET Tyr1003 13D11, rabbit monoclonal, #3135S	Cell Signaling/NEB, Frankfurt a.M.
Phospho-MET Tyr1234/1235 D26 XP, rabbit monoclonal, #3077S	Cell Signaling/NEB, Frankfurt a.M.
Phospho-p44/42 Erk1/2 T202/Y204, rabbit polyclonal, #9101S	Cell Signaling/NEB, Frankfurt a.M.

2.1.4 Therapeutics

Afatinib (BIBW2992), #SEL-S1011-5MG	Biozol, Eching
Cetuximab/Erbitux®™, 5 mg/ml	Merck KGaA, Pharmacy Klinikum rechts der Isar
Trastuzumab/Herceptin®™, 21 mg/ml	Roche Diagnostics, Pharmacy Klinikum rechts der Isar

2.1.5 Chemicals

Acetic acid, 100 %, #100063	Merck KGaA, Darmstadt
40 % Acrylamide/Bis Solution 37.5:1, #161-0148	BioRad, München
4´,6-Diamidin-2´-phenylindol-dihydrochlorid (DAPI), #10236276004	Sigma-Aldrich, Steinheim
Agarose, #840004	Biozym, Hessisch Oldendorf
Ammonium chloride, #254134	Sigma-Aldrich, Steinheim
Ammonium persulfate (APS), #A3678	Sigma-Aldrich, Steinheim
Aprotinin, #A6279-5ML	Sigma-Aldrich, Steinheim

BD Matrigel basement membrane matrix, #734-0270	VWR, Ismaning
Bovine Serum Albumin (BSA), #A7906 & #A7030 (ELISA)	Sigma-Aldrich, Steinheim
Bromophenol blue, #B0126	Sigma-Aldrich, Steinheim
Calcium chloride, #C5670	Sigma-Aldrich, Steinheim
Casein from bovine milk, #C5890-500G	Sigma-Aldrich, Steinheim
Citrate 20 mM pH3.0 sterile, #9871S	Cell Signaling/NEB, Frankfurt a.M.
Collagen type I, #734-1097	Corning/VWR, Ismaning
Coomassie Brilliant Blue #R-250	Serva Elecrophoresis, Heidelberg
p-coumaric acid, #065K1298	Sigma-Aldrich, Steinheim
Epidermal growth factor (EGF), human, #E9644-2MG	Sigma-Aldrich, Steinheim
Ethylenediaminetetraacetic acid (EDTA), #E9884-100G	Sigma-Aldrich, Steinheim
Fibrinogen, #F4883-500MG	Sigma-Aldrich, Steinheim
Fibronectin from bovine plasma, #F1141-1MG	Roche Diagnostics/Sigma-Aldrich, Steinheim
Gel Red Nucleic Acid Stain 10000x in Water, #41003-T	VWR, Ismaning
Generuler DNA ladder mix, #SM0331	ThermoFisher Scientific, Schwerte
Glycerin, 85 %, #104092	Merck KGaA, Darmstadt
Glycin p.a., 99 %, #50046	Merck KGaA, Darmstadt
Histogel™Richard-Allan Scientific™, #HG-4000-012	ThermoFisher Scientific, Darmstadt
Human Neuregulin-1 (hNRG-1), #5218SF	NEB, Frankfurt a.M.
Hydrogen peroxide, #31642	Sigma-Aldrich, Steinheim
N-(2-Hydroxyethyl)-piperazine, #H28807	Sigma-Aldrich, Steinheim
Isopropanol, #109634	Merck KGaA, Darmstadt
Potassium chloride, #104938	Merck KGaA, Darmstadt
Potassium hydrogen phosphate trihydrate, #105099	Merck KGaA, Darmstadt
Leupeptin trifluoroacetate, #L2023-1MG	Sigma-Aldrich, Steinheim
Luminol, #A8511	Sigma-Aldrich, Steinheim
Magnesium chloride, #814733	Merck KGaA, Darmstadt
Magnesium sulfate, #F00627-250G	Fluka Biochemika, Deisenhofen
2-Mercaptoethanol, ≥ 98 %, #M7154	Sigma-Aldrich, Steinheim
Methanol, #113351	Merck KGaA, Darmstadt

Milk powder 1KG, #CM35	Hartenstein, Würzburg
Sodium azide, #822335	Merck KGaA, Darmstadt
Sodium chloride, #567440	Merck KGaA, Darmstadt
Sodiumcitrat-dihydrate, #106448	Merck KGaA, Darmstadt
Sodium dodecyl sulfate (SDS) -Solution 20%, #A0675	AppliChem, Darmstadt
Sodium fluoride, #106450	Sigma-Aldrich, Steinheim
Sodium hydrogen phosphate dehydrate, #119753	Merck KGaA, Darmstadt
Sodium hydroxide, #109139	Merck KGaA, Darmstadt
Sodium orthovanadate, #450243	Sigma-Aldrich, Steinheim
Sodium phosphate, #342483	Sigma-Aldrich, Steinheim
Pepstatin A, #P5318-5MG	Sigma-Aldrich, Steinheim
Phenylmethanesulfonyl fluoride (PMSF), #P7626	Sigma-Aldrich, Steinheim
Protein Assay Dye Concentrate Reagent, #500-0006	BioRad, München
Protein Marker V, #27-2210	peqlab-VWR, Ismaning
Protein-Standard II (1,48 mg BSA/ml), #5000007	BioRad, München
Sea-Plaque Agarose, #849101	Biozym, Hessisch Oldendorf
TBE Buffer 10x, #15581028	Life Technologies, Darmstadt
Tetramethylethylendiamin (TEMED) 50ml, #161-0801	BioRad, München
Thrombin, #T4648-1MG	Sigma-Aldrich, Steinheim
Tris (Tris(hydroxymethyl)aminomethan), #75825	USB Europa, Staufen
Triton X-100, #112298	Merck KGaA, Darmstadt
TWEEN® 20, #822184	Merck KGaA, Darmstadt
Vectashield Mounting Medium 10ml, #VEC-H-1000	Biozol, Eching
Vitronectin human protein, #PHE0011	ThermoFisher Scientific, Darmstadt
Water, #116754	Merck KGaA, Darmstadt

2.1.6 Solutions and buffers

2.1.6.1 Buffers for protein isolation

10x L-CAM

Reagent	Concentration
NaCl	1.4 M
KCl	47 mM
$MgSO_4$	7 mM
$CaCl_2$	12 mM
Hepes	100 mM

1x L-CAM

Reagent	Volume
H_2O	635 µl
10 x L-CAM	100 µl
Triton X-100 (10 %)	10 µl
PMSF	20 µl
Na_3VO_4	20 µl
Aprotinin	12.7 µl
Leupeptin	2 µl
NaF	100 µl
$Na_4P_2O_2$ ' 10 H_2O	100 µl

5x Laemmli

Reagent	Concentration	Volume
1.25 M Tris-HCL pH 6.8	60 mM	2.5 ml
Glycerin	8.7 % (v/v)	4.5 ml
β-Mercaptoethanol	720 mM	2.8 ml
SDS	2 %	1 g
Bromphenolblue	by eye	

2.1.6.2 Buffers and solutions for western blot analysis

7.5 % separation gel (2 Mini-Gels 1.5 mm)

Reagent	Volume
H_2O	8.27 ml
1 M Tris, pH 8.8	3.75 ml
40 % AA	2.81 ml
20 % SDS	75 µl
10 % APS	90 µl
TEMED	9 µl

5 % stacking gel

Reagent	Volume
H_2O	3.05 ml
0.5 M Tris, pH 6.8	1.25 ml
40 % AA	0.63 ml
20 % SDS	25 µl
10 % APS	40 µl
TEMED	4 µl

10x SDS-PAGE Running buffer

Reagent	Volume
Glycine	14.4 g (1.92 M)
Tris	3.02 g (250 mM)
SDS	0.5 % (v/v)
H_2O	900 ml

adjust pH value to 8.8

1x SDS-PAGE Running buffer

Reagent	Volume
10x SDS-PAGE Running buffer	100 ml
H_2O	900 ml

10x Blotting buffer

Reagent	Volume
Glycine	14.4 g (1.92 M)
Tris	3.02 g (250 mM)
SDS	0.2 % (v/v)
H_2O	900 ml

1x Blotting buffer

Reagent	Volume
10x Blotting buffer	100 ml
Methanol	200 ml
H_2O	900 ml

10x TBS

Reagent	Volume
Tris Base	24.2 g (500 mM)
NaCl	80 g (1.5 M)
H_2O	1000 ml

adjust pH value to 7.3

1x TBS-T

Reagent	Volume
10x TBS	100 ml
TWEEN®20 (10 %)	10 ml
H_2O	890 ml

TBS-T 5 % MP (milk powder)

Reagent	Volume
Milk powder	2.5 g
1x TBS-T	50 ml

TBS-T 5 % BSA

Reagent	Volume
BSA	2.5 g
1x TBS-T	50 ml

ECL (enhanced chemiluminescence) reagent self-made

Solution 1	Reagent	Volume
keep dark	250 mM Luminol	1 ml
	100 mM p-Coumaric acid	400 µl
	1 M Tris-HCl pH 8.6	10 ml
	H_2O	88.6 ml

Solution 2	Reagent	Volume
	8.8 M H_2O_2 (30 %)	50 µl
	1 M Tris-HCl pH 8.6	10 ml
	H_2O	89.5 ml

Solutions were mixed 1:1 before using.

2.1.6.3 Buffers for ELISA

Washbuffer

Reagent	Volume
TWEEN®20	0.5 ml
1x PBS	1000 ml

adjust pH value to 7.2 - 7.4

Reagent Diluent

Reagent	Volume
BSA	0.5 g
1x PBS	50 ml

adjust pH value to 7.2 - 7.4, sterile filtrated (0.2 µm)

Substrate Solution

Reagent	Volume
Solution A	5 ml
Solution B	5 ml

Stop Solution

2N H_2SO_4

2.1.6.4 Buffers for DNA analysis

1x TBE

Reagent	Volume
10x TBE	100 ml
H_2O	900 ml

1.5 % Agarose gel

Reagent	Volume
Agarose	1.5 g
1x TBE	100 ml
Gel Red	5 µl

2.1.6.5 Other solutions

Cetuximab solvent

Reagent	Volume
NaCl	8.48 mg
Na_2HPO_4 ' 2 H_2O	1.88 mg
NaH_2PO_4 ' H_2O	0.42 mg
H_2O	1 ml

Freezing solution

Reagent	Volume
DMSO	100 µl
cell culture medium (full medium)	100 µl
FCS	120 µl

2.1.7 Consumables

Amersham Hybond PVDF, 0.45 µm, #1060023	GE Healthcare, München
Cell Scraper, 25 cm, #83.1830	Sarstedt, Nümbrecht
Cover slips 20*20, #DK20	Hartenstein, Würzburg
Cryo vials 2.0 ml, #89020	TPP/Faust Labscience, Klettgau
Eppendorf cup 0.5 ml, 1.5 ml, 2 ml	Eppendorf, Hamburg
Falcontube 15 ml, 50 ml	Falcon®/VWR, Ismaning
Filter, Millex® GP 0.22 µm	Merck KGaA, Darmstadt
Foam Pads - Pack of 4; 8*11 cm, #170-3933	BioRad, München
Hypercassette™ No.25902H 20x25 cm	Amersham/GE Healthcare, München
Neubauer chamber 0.0025 mm^2	Superior, Marienfeld
Parafilm®, M, PF-10	Hartenstein, Würzburg
PCR SingleCap 8er-SoftStrips 0.2 ml, #710971	Biozym, Hessisch Oldendorf
PE-tube 300 mm/100 µm, 25 m, #PE30	Hartenstein, Würzburg
Pipettes	
costar Stripette 5 ml, 10 ml, 25 ml	corning/Sigma-Aldrich, Steinheim
disposable Pasteur pipettes 3 ml	GLW, Würzburg
Serological, #710180/2 ml; # 606180/5 ml; #607180/10 ml; #768180/25 ml	Cellstar® greiner bio-one, Frickenhausen
Pipette tips, 2-20 µl, 20-200 µl, 100-1000 µl	TipONE starlab, Hamburg
Scalpel	Sapphire, Pocasset, USA

Syringe, 20ml, #6702740	B-Braun/Faust Labscience, Klettgau
Syringe filter, Minisart 0.2 µm #FI02	Hartenstein, Würzburg
Tissue culture dish	
22.1 cm^2,#93060	TPP/Faust Labscience, Klettgau
60.1 cm^2,#93100	TPP/Faust Labscience, Klettgau
147.8 cm^2,#93150	TPP/Faust Labscience, Klettgau
6-well plates, #92006	TPP/Faust Labscience, Klettgau
12-well plates, #92012	TPP/Faust Labscience, Klettgau
24-well-Companion Plate, #734-0067	VWR, Ismaning
96-well, 0.335cm^2, #92096	TPP/Faust Labscience, Klettgau
Corning Costar Ultra-Low 24-well, #CLS3473	Sigma-Aldrich, Steinheim
Corning Costar Ultra-Low 24-well round bottom, #CLS7007-24EA	Sigma-Aldrich, Steinheim
Glass bottom 35 mm P35G-0-14-C	MatTek Corporation, Ashland, USA
Tissue culture flask	
25 cm^2, #90026	TPP/Faust Labscience, Klettgau
75 cm^2 ,#90076	TPP/Faust Labscience, Klettgau
Whatman® GB005	Biometra, Göttingen
X-Ray Film Fuji Super RX blau, #RF12	Fuji/ Hartenstein, Würzburg

2.1.8 Commercial Kits

AllStars Negative Control siRNA (5 nmol), #1027280	Qiagen, Hilden.
AllStars Neg. siRNA AF 488 (5 nmol), #1027284	Qiagen, Hilden
BioCoat Matrigel Invasions Chambers, 8µm, #734-1047	Corning/VWR, Ismaning
Cell proliferation reagent WST-1, #11644807001	Sigma-Aldrich, Steinheim
Control cell culture inserts, 8µm, #734-0221	Corning/VWR, Ismaning
FlexiTube GeneSolution ERBB3 1027416, #GS2065	Qiagen, Hilden
FlexiTube GeneSolution AXL 1027416, #GS558	Qiagen, Hilden
Human ErbB2/Her2 DuoSet ELISA, #DY1129B	R&D/Bio-Techne, Wiesbaden
Lipofectamine 2000® Transfection Reagent 1.5ml, #11668019	ThermoFisher Scientific, Schwerte
PCR Mycoplasma Test Kit I/C, #PK-CA91-1048	PromoCell, Heidelberg

Proteome Profiler Human Phospho-Kinase Array Kit, #ARY003B	R&D/Bio-Techne, Wiesbaden
Proteome Profiler Human Phospho-RTK Array Kit, #ARY001B	R&D/Bio-Techne, Wiesbaden
Substrate Reagent Pack (8 vials Color A, 8 vials Color B), #DY999	R&D/Bio-Techne, Wiesbaden

2.1.9 Equipment

Autoclave	
Varioklav®EC Steam Sterilizer	H+P, Oberschleißheim
Centrifuge	
Universal 320	Hettich Zentrifugen, Tuttlingen
Micro-Centrifuge SD 220VAC	Roth, Karlsruhe
4K15	Sigma-Aldrich, Steinheim
Electrophoresis Cell	
Mini-Cell Xcell SureLock™, #100901-845	Invitrogen™ Novex®/Thermo Scientific, Darmstadt
Electrophoresis Chamber	
Wide Mini-Sub® Cell GT System, #170-4468	Biorad, München
ELISA Reader, Expert Plus	Asys, Eugendorf, Österreich
Film Processor	
Konica SRx-101A	Konica Minolta, Langenhagen
Freezer -86°C HeraFreeze Basic, #51019576	Thermo Scientific, Darmstadt
Fridge	Liebherr, Kirchdorf
Gelstation E-box VX2	Vilber, Eberhardszell
Heating block 20-120°C	Bachofer
Laminar flow Hood S@feflow 1.2	nunc™, Wiesbaden
Incubation System	
Incubation System 2	Zeiss AG Jena
Heating insert, #411861-9901-000	Zeiss AG Jena
Incubator S, #411860-9902-000	Zeiss AG Jena
Tempcontrol 37-2, #1052320	Zeiss AG Jena
CTI-Controller 3700, #411856-9903	Zeiss AG Jena
Incubator #9140-0052	Binder, Tuttlingen
Microscope	
Labovert FS	Leitz, Wetzlar
Axio Observer.A1, #3832001309	Zeiss AG Jena

Mini PROTEAN® System	
Spacer 1.5 mm Glass Plates, #1653312	Biorad, München
1.5 mm Short Plates, #1653308	Biorad, München
1.5 mm 10 Well Comb, #1653365	Biorad, München
Electrophoresis system	Biorad, München
Mini Trans®-Blot cell	Biorad, München
Electrophoretic transfer cell #1703930	Biorad, München
Nano Drop® 1000	PeqLab Biotechnologie, Erlangen
pH meter, 211 Microprocessor	HANNA Instruments, Woonsocket, USA
Pipetman 0,2 – 2 µl, 2 – 200 µl, 20 – 1000µl	Gilson International, Villiers le Bel, France
pipetus® rechargeable pipette controller	Hirschmann/Sigma-Aldrich, Steinheim
Power Pac™ Basic, #041BR93468	Biorad, München
Roller RM5 Assistant	Karl Hecht GmbH, Sondheim vor der Rhön
Scale, #BP310S	Sartorius-Stedim, Göttingen
Sealer, Impulse E82163(S) TISH-300	TEW, Northridge, USA
Rocking Platform STRS	Stuard Scientific, Oregon, USA
Test-tube rotator LD79, #79000	Labinco BV, Breda, Netherlands
Thermal Cycler	
GeneAmp® PCR System 9700	Applied Biosystems®/Thermo Scientific, Darmstadt
Waterbath	
37°C, #1012	GFL, Burgwedel

2.1.10 Software

AxioVision Rel 4.8	Zeiss AG Jena
Endnote	Thomson Reuters, Carlsbad, USA
Image J 1.47v	National Institute of Health
LSM Image Browser 4.2.0.121	Zeiss AG Jena
Microsoft Office 2016	Microsoft, Redmond, USA
IBM SPSS Statistics 23	SPSS Inc., Chicago, USA

2.2 Methods

2.2.1 Cell culture

All cell culture work was performed under a laminar flow hood using sterile flasks, plates, pipettes and solutions. Cells were maintained in a humidified atmosphere at 37°C, 5 % CO_2 and 95 % O_2. Different cell culture media were used, appropriate for the individual gastric cancer cell lines (Table 1).

Table 1: Cell culture media with additives for different gastric cancer cell lines.
For the individual gastric cancer cell lines different media were necessary. The culture media were supplemented with different additives.

Cell culture medium	Additives	Cell line
DMEM + GlutaMAX-I	10 % FCS, 1 % Pen/Step	Hs746T HSC-45
Minimum Essential Medium Eagle	10 % FCS, 1 % Pen/Step	HGC-27
Nutrient mixture F-10 Ham	10 % FCS, 1 % Pen/Step	LMSU
RPMI 1640 Medium	10 % FCS, 0.5 % Pen/Step, 2 mM L-Glutamine	AGS GSU H-111-TC KATO-III MKN1 MKN7 MKN45 NCI-N87

2.2.1.1 Thawing and freezing

For long-term storage, cell aliquots were frozen in liquid nitrogen. In order to start culturing a cell line, a frozen cell aliquot was thawed for 1 minute in a water bath at 37°C. Afterwards, 500 µl of preheated cell culture medium was added to the cryo vial. The cell suspension was transferred into a 25 cm^2 cell culture flask containing 5 ml of preheated cell culture medium. The cell culture medium was exchanged the following day.

To freeze cells for storage, a confluent flask of cells was needed. Cells were detached (see ”Passaging”) and 1 ml of the cell suspension (guide number: 10^6 cells) was added to the self-made freezing solution. The cryo vial was quickly frozen at -80°C and subsequently stored in liquid nitrogen.

2.2.1.2 Passaging

After the cells had reached an appropriate degree of confluence for desired experiments or for further cell culture, the medium was discarded with a vacuum pump. To remove the remaining serum-containing cell culture medium, the cells were washed with PBS. To detach the adherent cells from the cell culture dish, the cells were incubated with Trypsin-EDTA (0.05 %) or with Versene for MKN7. After incubating for an appropriate amount of time at 37°C, the cells were disaggregated with fresh culture medium and transferred into a fleshly prepared flask. For further experiments, the number of cells was determined.

2.2.1.3 Determination of the cell number

For all experiments, the cell number of detached and disaggregated cells was determined with a Neubauer chamber. To count the cells, 10 µl of the cell suspension were transferred into a prepared chamber. The cells in four major squares of the Neubauer chamber were counted, in order to calculate the cell number, as shown below.

$$\frac{Total\ \text{counted}\ number\ of\ cells}{4}\ \text{x}\ 10^4\ \text{x}\ \ dilution\ factor = \frac{Number\ of\ cells}{ml}$$

$$\frac{Number\ of\ cells}{ml}\ \text{x applied cell culture medium [ml]} = \text{Total number of cells}$$

2.2.1.4 Mycoplasma PCR

2.2.1.4.1 Sampling

To test the thawed cell lines for *Mycoplasma*, 1 ml supernatant of an 80 % confluent cell culture flask was transferred into an Eppendorf cup. This test sample was denatured for 5 min at 95°C, centrifuged and chilled on ice. Corresponding fresh culture medium was processed equally, serving as a negative control.

2.2.1.4.2 PCR

The lyophilized components of the tubes in the *PCR Mycoplasma Test Kit I/C* needed to be rehydrated by adding 23 µl of Rehydration Buffer. Cell culture medium for the negative control, supernatant for the test sample and DNA-free water for the positive control (red tube), each 2 µl were added. After incubation at RT for 5 min, the prepared samples were placed in the thermal cycler, running the following program.

Time	Temperature	
2 min	95°C	
30 sec	94°C	} 40 cycles
30 sec	55°C	
40 sec	72°C	
> 10 min	4°C	

2.2.1.4.3 Agarose gel electrophoresis

To analyze the generated DNA, a 1.5 % agarose gel was prepared. To completely dissolve the agarose, the mixture was heated in a microwave. For staining, 5 µl Gel Red Nucleic Acid Stain were added and the solution was poured into the gel device. After the gel had polymerized completely, 4 µl Generuler DNA ladder mix and 8 µl of the samples were loaded. The electrophoresis ran at 110 V, 30 min in 1x TBE. To visualize the DNA, the gel was analyzed under UV-light. The signal for the negative control and negative samples was expected at 479 bp, indicating a successfully performed PCR. Whereas for the positive control and positive sample, bands were expected at 270 bp and 479 bp.

2.2.2 Protein analysis

2.2.2.1 Stimulation

To analyze the molecular effect of trastuzumab in the different gastric cancer cell lines, the cells were treated with 5 µg/ml trastuzumab for different time periods.

For every sample, an appropriate number of cells was seeded depending on the cell culture dish and cell line (Table 2). The next day, the culture medium was exchanged and after a resting period of 2 h, the cells were treated with 5 µg/ml trastuzumab. At the desired time point, the sample were prepared. Treatment time periods were 5 min, 10 min, 20 min, 30 min, 1 h, 4 h, 7 h, 22 h, 24 h or 72 h, as indicated in the relevant experiments.

Table 2: Cell numbers for seeding.
Depending on the growing surface of cell culture plates different amounts of cells needed to be seeded. Thus, the volume of applied lysis buffer was altered. For the cell line NCI-N87 more cells needed to be seeded than for other cell lines.

Cell number	Dish	L-CAM
$2.5 * 10^6$ ($2.9 * 10^6$ for NCI-N87)	15 cm (147.8 cm^2)	200 µl
$1.0 * 10^6$ ($1.2 * 10^6$ for NCI-N87)	10 cm (60.1 cm^2)	120 µl
$3.7 * 10^5$ ($4.4 * 10^5$ for NCI-N87)	6 cm (22.1 cm^2)	60 µl
$1.5 * 10^5$ ($1.8 * 10^5$ for NCI-N87)	well/6-well plates	60 µl
$5.8 * 10^4$ ($6.9 * 10^4$ for NCI-N87)	well/12-well plates	60 µl

2.2.2.2 Sample preparation

To generate samples for western blot or proteome profiler analysis the medium of the cells was discarded and washed two times with ice-cold PBS. Subsequently, 1x L-CAM or lysisbuffer from the proteome profiler kit, was added. The cells were scraped off into an Eppendorf cup. The sample was vortexed, centrifuged at 4°C, 12,000 rpm for 10 min and the supernatant transferred into a new Eppendorf cup.

2.2.2.3 Bradford assay

To determine the protein concentration of the samples a Bradford assay was performed in a 96-well plate. For every experiment a standard curve was generated, diluting Protein-Standard II (1:200 in H_2O), in serial steps with Protein Assay Dye Reagent (1:5 in H_2O) (0, 1.48, 4.44, 7.4, 10.36 µg/ml). For measuring of the sample, 1 µl of cell supernatant was mixed with 199 µl of the diluted Protein Assay Dye Reagent in triplicates. The plate was vortexed and incubated for 5 min. Absorbance was measured with an ELISA reader at 595 nm. A standard curve was created by linear regression analysis and the sample concentration was calculated.

2.2.2.4 Western blot analysis

Different proteins can be separated by size, moving to the anode during the sodiumdodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE). Therefore, the protein samples were mixed 1:5 with 5x Laemmli, denatured for 5 min at 95°C and chilled on ice. The 5x Laemmli includes 2-mercaptoethanol, which cleaves the disulfide bonds. SDS ensures the denaturation of the protein and attaches negative charges.

2.2.2.4.1 SDS PAGE

For the electrophoresis, 7.5 % SDS-gels were used, consisting of a separation gel and a stacking gel. The separation gel was prepared, poured between two glass plates, fixed in the gel-casting device and overlaid with isopropanol. After polymerization, the isopropanol was removed, and the prepared stacking gel was poured onto the separation gel, inserting a 15-well comb immediately. After polymerization, the gel was stored at 4°C (maximum 2 days) or directly placed into the electrophoresis chamber. The pockets of the gel were purged and loaded with 25 µg protein or 3 µl Protein Marker V. The chamber was filled with 1x SDS-PAGE Running buffer and the electrophoresis was set at 110 V for 75 min.

2.2.2.4.2 Blotting

The proteins, separated by SDS-PAGE, were transferred onto a PVDF membrane using a wet blotting system. A sandwich system was applied in the following order: sponge, 2 Whatman®, SDS-gel, PVDF membrane, 2 Whatman®, sponge. Before using, the PVDF membrane was activated with methanol. The system and an ice pack were placed into the blotting chamber and filled with 1x Blotting buffer. The blotting was performed at 110 V for 75 min.

2.2.2.4.3 Immunostaining

After the blotting, the membrane was blocked with TBS-T 5 % MP for 1 h to prevent unspecific signals. The first antibody was incubated overnight at 4°C, diluted in TBS-T 5 % MP or TBS-T 5 % BSA. Different antibody concentrations were tested, best conditions are shown in Table 3. The following day, the membrane was washed three times with 1x TBS-T and incubated with the second HRP-linked antibody for 1 h. After this incubation the membrane was washed three times with 1x TBS-T. The detection reaction was initiated with ECL reagent self-made. The membrane was transferred into a cassette and exposed to chemiluminescent films for various time periods, depending on the signal intensity.

Table 3: Antibody dilutions and expected signals.
Every applied antibody was diluted before using to obtain the best signalintensities. The antibodies were diluted in TBS-T 5 % MP, TBS-T 5 % BSA or 1x TBS-T. Depending on the analyzed protein, the signal was detected on the membrane at the corresponding size.

Target	Dilution	Signal
α-tubulin	1:10000 TBS-T 5 % MP	50 kDa
β-actin	1:10000 TBS-T 5 % MP	42 kDa
anti-mouse, HRP-linked	1:10000 TBS-T 5 % MP	depending on the first antibody
anti-rabbit, HRP-linked	1:2000 1x TBS-T	depending on the first antibody
AXL	1:1000 TBS-T 5 % BSA	138 kDa
EGF Receptor	1:1000 TBS-T 5 % MP	175 kDa
HER2/ErbB2 29D8	1:1000 TBS-T 5 % BSA	185 kDa
HER2/ErbB2 CB11	1:10000 TBS-T 5 % MP	different (185, 140, 110, 95 kDa)
HER2/ErbB2 e2-4001 + 3B5	1:2000 TBS-T 5 % BSA	different (185, 140, 110, 95 kDa)
HER3/c-erbB-3	1:1000 TBS-T 5 % BSA	185 kDa
HER3/ErbB3 1B2	1:1000 TBS-T 5 % BSA	185 kDa
MET	1:1000 TBS-T 5 % BSA	145 kDa
Phospho-Akt S473	1:2000 TBS-T 5 % BSA	60 kDa
Phospho-Akt S473 XP	1:2000 TBS-T 5 % BSA	60 kDa
Phospho-AXL Y702	1:500 TBS-T 5 % BSA	138 kDa

Phospho-EGFR Y1068	1:2000 TBS-T 5 % MP	185 kDa
Phospho-HER2/ErbB2 Y1221/1222	1:1000 TBS-T 5 % BSA	185 kDa
Phospho-HER2/ErbB2 Y1248	1:1000 TBS-T 5 % BSA	185 kDa
Phospho HER3/ErbB3 Y1222	1:1000 TBS-T 5 % BSA	185 kDa
Phospho-HER3/ErbB3 Y1289	1:1000 TBS-T 5 % BSA	185 kDa
Phospho-MET Y1003	1:1000 TBS-T 5 % BSA	145 kDa
Phospho-MET Y1234/1235	1:1000 TBS-T 5 % BSA	145 kDa
Phospho-p44/42 Erk1/2 T202/Y204	1:2000 TBS-T 5 % MP	42/44 kDa

2.2.2.4.4 Quantitative analysis of proteins

For the quantification of the signals, a densitometric analysis was performed using ImageJ 1.47v Software. Therefore, the chemiluminescent films were scanned with a resolution of 300 dpi and saved as TIFF file. The intensity of the signals was measured, making sure to exclude overexposed signals. For normalization of the data, a housekeeping protein (α-tubulin or β-actin) was analyzed additionally.

2.2.2.5 Proteome Profiler analysis

To analyze the activation status of many proteins at the same time in one sample, a proteome profiler is an efficient method. To decide which treatment condition or treatment combinations would be the most reasonable to analyze with the proteome profiler, Dr. S. Keller performed western blot analyses with different compounds and treatment durations. NCI-N87 cells were treated with trastuzumab plus/or afatinib, cetuximab, lapatinib, pertuzumab and analyzed for the activation of EGFR, HER2, and HER3, and the kinases ERK and AKT. Furthermore, the most suitable treatment time was determined for every compound. The inhibitory effect of trastuzumab plus/or afatinib for 20 minutes and the stimulative effect of the ligands EGF and NRG on the activation of kinases for 5 minutes was analyzed in the gastric cancer cell lines MKN1, MKN7 and NCI-N87.

Two different proteome profilers were executed. The Human Phospho-Kinase Proteome Profiler detects the levels of 43 specific kinase phosphorylation sites and 2 related total proteins. The Human Phospho-RTK Proteome Profiler analyzes the tyrosine phosphorylation of 49 receptor tyrosine kinases. Treatment conditions differed between the two proteome profilers as shown in Table 4 and Table 5. The preparation of the samples was done as described in “Sample preparation”. Experiments were performed in triplicate according to the manufacturer’s instructions. Lysates were diluted in blocking buffer with a total protein amount of 300 µg per array in the Phospho-RTK Array and 400 µg in the Phospho-Kinase Array. The preblocked membranes

were incubated overnight. After repeating washing steps, the membranes were incubated in the provided detection antibody for 2 h. After additional washing steps the membranes were incubation in streptavidin-horseradish peroxidase (HRP) for 30 min. Unbound HRP-antibody was removed by washing before the signals were analyzed by a chemiluminescent substrate and detected by chemiluminescent films. For quantification, the signals were densitometrically analyzed using ImageJ and statistical analysis was done and described by Dr. J. Hasenauer as follows.

The measured proteome profiler data were log2-transformed to improve the normality and analyzed using the MATLAB statistical toolbox. For each phosphorylated protein, a linear mixed model was inferred to evaluate the treatment response, the batch effect and the noise level. This yielded fold-changes and p-values for each phosphorylated protein and each pair of treatments. The p-values were adjusted using the Bonferroni-Holm correction for multiple testing. For visualization purposes, the adjusted p-values were categorized (≤ 0.001; 0.001 - 0.01; 0.01 - 0.05), and only significant changes with (i) a fold-change > 1.5 and (ii) an average control greater than twice the array internal negative control were considered. This work has been published by Keller, Zwingenberger and colleagues (Keller et al. 2018).

Table 4: Generated cell culture samples for the Phospho-RTK Proteome Profiler.
For the Phospho-RTK Proteome Profiler samples of MKN1, MKN7 and NCI-N87 were generated in triplicates. The cells were treated for 20 minutes with 5 µg/ml trastuzumab, 0.5 µM afatinib, 5 ng/ml EGF or the combinations.

Cell lines	Treatment	Time
MKN1, MKN7 and NCI-N87	untreated	-
	5 µg/ml trastuzumab	20 min
	0.5 µM afatinib	20 min
	5 ng/ml EGF	20 min
	5 µg/ml trastuzumab + 5 ng/ml EGF	20 min
	0.5 µM afatinib + 5 ng/ml EGF	20 min
	5 µg/ml trastuzumab + 0.5 µM afatinib	20 min
	5 µg/ml trastuzumab + 0.5 µM afatinib + 5 ng/ml EGF	20 min

Table 5: Generated cell culture samples for the Phospho-Kinase Proteome Profiler.
For the Phospho-Kinase Proteome Profiler samples of MKN1, MKN7 and NCI-N87 were generated in triplicates. The cells were treated for 20 min with 5 µg/ml trastuzumab, 0.5 µM afatinib or the combination of 5 µg/ml trastuzumab plus 0.5 µM afatinib. Other samples were treated for 5 min with 5 ng/ml EGF or 20 ng/ml NRG1.

Cell lines	Treatment	Time
MKN1, MKN7 and NCI-N87	untreated	-
	5 µg/ml trastuzumab	20 min
	0.5 µM afatinib	20 min
	5 µg/ml trastuzumab + 0.5 µM afatinib	20 min
	5 ng/ml EGF	5 min
	20 ng/ml NRG1	5 min

2.2.2.6 HER2 shedding

As described, HER2 shedding is a resistance mechanism occurring in tumors overexpressing HER2. The HER2 shedding was analyzed by ELISA and an antibody directed against the c-terminal domain of HER2 in the cell lines MKN1, MKN7 and NCI-N87. All cell lines were treated as shown in Table 6. In order to determine the effects of culturing, samples were generated of untreated cultures at different time points. The additional treatment of the cells with 10 µg/ml trastuzumab or 1 mM pervanadate was intended for a more complex analysis of the HER2 shedding. At the indicated time points, the supernatant was collected for ELISA. Furthermore, total lysates were prepared for protein analysis by western blot analysis.

Table 6: Generated samples for the analysis of HER2 shedding.
Samples for the analysis of HER2 shedding were generated for MKN1, MKN7 and NCI-N87 in triplicates. The cells were treated for different time periods with 5 µg/ml trastuzumab. For three of these time periods, the samples were treated with 10 µg/ml trastuzumab. Pervanadate was used to promote stress and therefore possible HER2 shedding in the cell lines.

Treatment	Concentration	Duration
-		0 h
-		4 h
-		22 h
-		72 h
trastuzumab	5 µg/ml	5 min
trastuzumab	5 µg/ml	30 min
trastuzumab	5 µg/ml	1 h
trastuzumab	5 µg/ml	4 h
trastuzumab	5 µg/ml	7 h
trastuzumab	5 µg/ml	22 h
trastuzumab	5 µg/ml	72 h
trastuzumab	10 µg/ml	5 min

trastuzumab	10 µg/ml	4 h
trastuzumab	10 µg/ml	72 h
pervanadate	1 mM	1 h
pervanadate	1 mM	4 h

2.2.2.6.1 Enzyme-linked immunosorbent assay (ELISA)

For ELISA, the supernatants of the cell samples were collected to determine the concentration of the HER2 ECD. The commercial ELISA Kit Human ErbB2/Her2 only detects HER2 ECD. The supernatants were transferred into Eppendorf cups and stored at -80°C. Experiments were performed in biological and technical triplicates according to the manufacturer's instruction protocol. Briefly, a 96-well plate was coated with a capture antibody (working concentration: 2 µg/ml) and incubated overnight. After several washing steps with Washbuffer, the plate was blocked with Reagent Diluent and incubated for 1 h, followed by additional washing steps with Washbuffer. The test samples and a standard curve (0, 54.7, 109, 219, 438, 875, 1750, 3500 pg/ml), diluted in Reagent Diluent were loaded and incubated for 2 h. After several more washing steps, the plate was incubated with the provided detection antibody (working concentration: 0.1 µg/ml) for 2 h, followed by washing steps and a incubation in streptavidin-HRP for 20 min. Three more washing steps followed, before incubating the plate with Substrate Solution for 20 min and adding Stop Solution. The plate was gently tapped and optical density was measured with an ELISA reader at wavelength/wavelength correction 450 nm/550 nm. The standard curve was created by linear regression analysis and the concentration of HER2 ECD in the samples was calculated.

2.2.2.6.2 Analysis of the intracellular domain of HER2

For the analysis of the intracellular domain of HER2 the corresponding lysates to the ELISA samples, were generated. The protein expression was analyzed via western blot analysis as described in "Western blot analysis". The applied antibody (ErbB2 Monoclonal Antibody (e2-4001 + 3B5) detects all membrane-bound forms of HER2, by binding to an intracellular domain of HER2, thus the shedded forms of HER2 in the cell lines can be analyzed.

2.2.3 Phenotypic analysis

2.2.3.1 Invasion analysis

To analyze the invasive potential of the different gastric cancer cell lines and the effect of trastuzumab, a commercial invasion assay was performed. In this commercial assay the

invasiveness was analyzed with a Matrigel®-covered membrane, which the cells needed to overcome by invasion and migration. The experiments were performed in biological triplicates according to the manufacturer's instructions, visualized in Figure 8.

The invasion-inserts were chilled to room temperature and placed in a 24-well plate. The inserts were rehydrated 2 h at 37°C, filling the wells and the inserts with 500 µl starvation medium (without FCS). Fresh starvation medium was added to the wells and 2 * 10^4 cells in starvation medium were transferred into the inserts. After 4 h, the medium in the inserts was replaced by fresh starvation medium with or without 5 µg/ml trastuzumab. In the wells starvation medium plus 0.1 % FCS was transferred, serving as a chemoattractant. The media were discarded after 22 h and the inside of the inserts were scrubbed with a damped cotton swab. The inserts were washed with PBS (with Mg^{2+} & Ca^{2+}) and scrubbed again. Following, the inserts were incubated in a previously prepared DAPI (4´,6-Diamidin-2´-phenylindol-dihydrochlorid) solution (0.05 µg/ml in methanol) in the dark for 15 min, staining the positive invasive cells resp. the nuclei of the cells. Afterwards, the inserts were washed with PBS (with Mg^{2+} & Ca^{2+}) and H_2O. The membrane on the bottom of the insert was removed with a scalpel, sealed on a slide and stored at -20°C. For analysis, UV-light plus DAPI-filter was used to microscopically count the fluorescent cells in five visual fields.

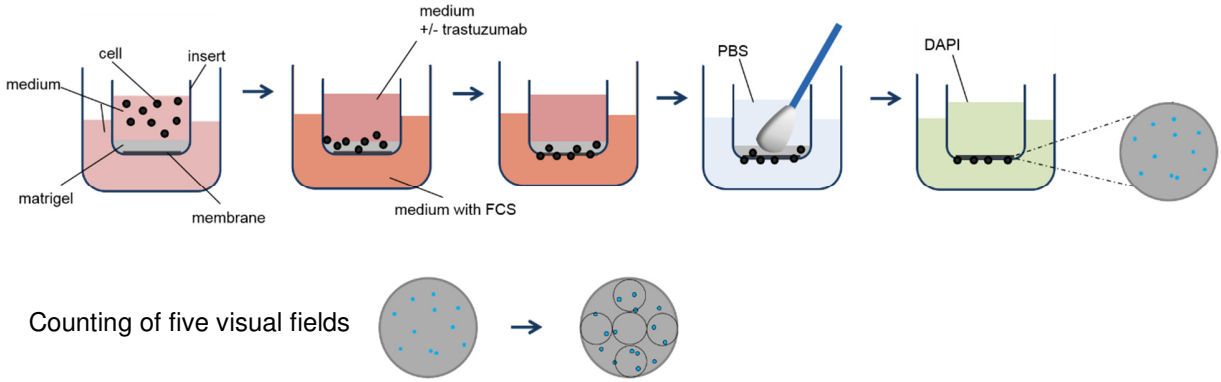


Figure 8: Scheme of the workflow of the invasion analysis.
The invasiveness of different gastric cancer cell lines was analyzed with a commercial invasion assay. Cells were seeded and treated with or without 5 µg/ml trastuzumab. FCS was applied as an attractant. Positive invasive cells were stained with DAPI and counted with a microscope filter set.

2.2.3.2 Motility analysis

2.2.3.2.1 Experimental setup

The motility of cells was analyzed by setting up a time-lapse microscopy and analyzing the movement of the cells for 7 h in the obtained film.

Before starting the motility analysis, the ideal cell number and coating for each cell line needed to be determined. Therefore, different amount of cells, different coatings and coating concentrations were tested. As coatings, collagen type I, fibronectin, laminin and Matrigel® were analyzed in

different concentrations. For the analysis, a cell culture dish with a glass bottom was washed with PBS, following the coating. After polymerization, the dish was washed with PBS again. Cells were seeded in the determined cell number and incubated 1 h (24 h for MKN7) at 37°C. Before starting the film, the medium was exchanged with freshly prepared full medium, with or without trastuzumab treatment. The dish was placed in the Incubation System and the reservoirs filled up with H_2O. The temperature was set to 37°C and 5 % CO_2. The motility was recorded for 8 h, adjusting the contrast in the first hour, analyzing the following 7 h.

2.2.3.2.2 Coatings

Fibronectin was used as coating at a concentration of 10 µg/ml in H_2O for all gastric cancer cell lines. After a polymerization for 90 min at RT the cells were seeded.

For collagen type I, different concentrations were analyzed (50, 75, 100, 130, 150 µg/ml in H_2O + acetic acid). A polymerization for 30 min at 37°C was necessary.

Laminin was analyzed as coating with a concentration of 1.65 µg/cm² in PBS. The stock solution needed to be thawed on ice and processed with pre-cooled material. An incubation for 2 hours at 37°C was necessary to ensure polymerization.

Another tested coating was Matrigel®. Matrigel® needed to be processed on ice with pre-cooled material. A concentration of 4 mg/ml in serum-free medium was analyzed after a polymerization for 1 h at RT.

2.2.3.2.3 Analysis of the motility and speed

The analysis of the motility and speed was performed manually with the LSM Image Browser. Cells, showing cell division, cell death or cells leaving the image frame during the film were excluded. Cells were determined as motile, when leaving their area, covered at the beginning, completely during the film. The outlines of the cells were mapped into the start-frame. Each cell was traced the throughout the film. Speed was determined as the distance of a cell, divided by the film length. For the analysis of further parameters, films were provided to Dr. J. Mattes, MATTES Medical Imaging GmbH, Hagenberg.

2.2.3.3 Proliferation analysis

To determine the proliferation and viability of gastric cancer cells a cell proliferation kit (WST-1) was performed. The stable tetrazolium salt WST-1 is cleaved to soluble formazan by a complex cellular mechanism. This mechanism is depending on the glycolytic production of NAD(P)H in

viable cells. Thus, the amount of formazan dye, measured by absorbance, directly correlated with the number of viable cells.

The sensitivity to trastuzumab was analyzed under different treatment concentrations (0, 0.1, 1, 10, 20, 40 µg/ml. The WST-1 assay was performed according to the manufacturer's instructions. All samples were analyzed in biological and technical triplicates. Cells were seeded in a 96-well plate at densities between 5 * 10^2 and 5 * 10^3 cells per well and allowed to settle for 24 h. The next day trastuzumab was added. The isotype-control was added to a final concentration of 40 µg/ml for trastuzumab. Furthermore, the effect of the isotype-solvent for trastuzumab was analyzed. After an incubation period of 72 h, pre-warmed WST-1 reagent was added. Depending on the cell line, the absorbance of the sample was measured after an incubation period between 30 min and 2 h. The absorbance was measured with an ELISA reader at wavelength/wavelength correction of 450 nm/620 nm.

2.2.4 Generation of formalin-fixed paraffin-embedded (FFPE)-cell pellets

To get a better comparability of the cell lines with patient samples, the cells were formalin-fixed and paraffin-embedded as performed with tumor tissue. The determination of the HER2 expression was performed at the University Cancer Center Leipzig (UCCL), as it is done with patient samples. All gastric cell lines from the project panel were seeded in three 15 cm cell culture dishes with a cell density of 2.5 * 10^6 cells (2.9 * 10^6 for NCI-N87). The following day, the medium was renewed. Depending on the desired cell pellet, different working steps were performed. For a regular untreated cell pellet, the cells were cultured for two more hours in the renewed medium. The antibody treated cell pellets were cultured for two more hours in the fresh medium, following 4 h of treatment with 1 µg/ml cetuximab or 5 µg/ml trastuzumab. For an untreated cell pellet, which was used as a control for the antibody treated cell pellet, the cells were cultured for six more hours in the renewed medium. Independent of the sample all cells were washed two times with PBS and scraped into a falcon tube. Next, 4 % formalin was added to the cells. The tube was inverted to assure good mixing. The formalin-fixed cells were stored overnight at RT and centrifuged the next day for 7 min, 500 rpm at 4°C. The supernatant was discarded and the cell pellet was resuspended in a small volume of remaining formalin. The cell suspension was transferred into a special system with a short tubular being fixed on a slide (Figure 9). The system was centrifuged for 7 min, 500 rpm at 4°C. The maximum of the supernatant was removed with a pipette. Heated Histogel™ (5 drops) was added on top of the cell pellet. After 30 min in the freezer the system was opened and the cell/HistoGel™ block was transferred into a labelled capsule. The capsule was stored in 4 %

formalin until it was grouted with paraffin by technicians, sliced and hematoxylin and eosin (H&E) stained. These H&E staining were reviewed and evaluated on their quality by PD Dr. med. G. Weirich. Afterwards they were provided to the university cancer center Leipzig (UCCL) for the determination of HER2 expression by IHC and *HER2* amplification by chromogenic in situ hybridization (CISH). Three biological independent samples of each cell line were prepared by K. Ebert, S. Keller and me.

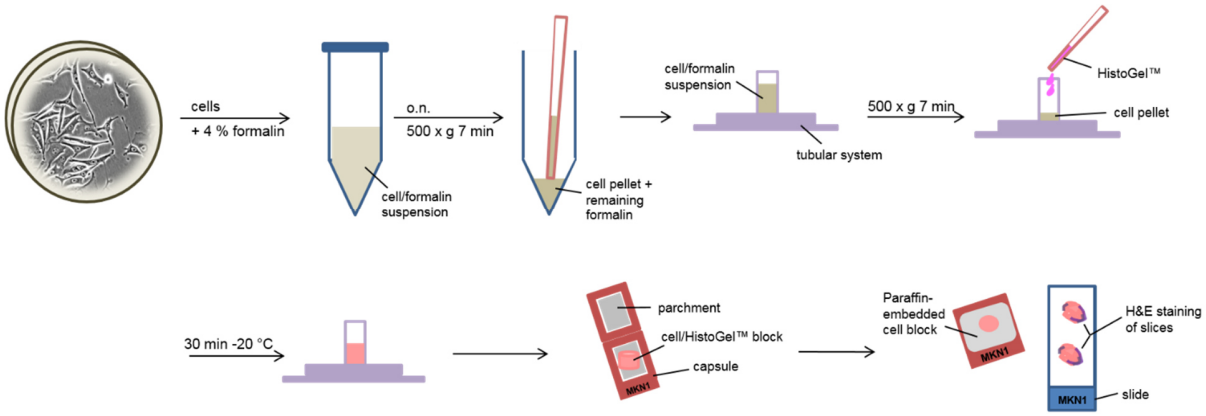


Figure 9: Scheme of the workflow of the FFPE-pellet generation.
FFPE-cell pellets were generated for all cell lines of the gastric cancer cell line panel. Cells were treated with 1 µg/ml cetuximab or 5 µg/ml trastuzumab. Cells were fixed with 4 % formalin and paraffin embedded. The samples were sliced and H&E stained for the evaluation of their quality.

2.2.5 siRNA Transfection

To generate a transient knockdown of a specific protein, cells were transfected with small interfering RNA (siRNA). To analyze the effect of AXL in the cell line MKN7, the cell line was siRNA transfected. Cells were seeded in a 6-well plate in 2 ml full medium and cultured overnight. The medium was exchanged for medium without the antibiotics Pen/Strep, to prevent cell death during siRNA uptake (Thermo Fisher Scientific Inc 2015). A specific FlexiTube GeneSolution was used, containing four gene specific, preselected siRNAs. The transfection reagent lipofectamine 2000® (5 µl) was diluted with Opti-MEM (250 µl) and incubated for 5 min. The oligomers were diluted in different concentrations with Opti-MEM. Both solutions were mixed and incubated for 20 min, before being transferred to the cells. This incubation is necessary for the formation of cationic liposomes and their uptake of the siRNAs. After a transfection time of 4 h, the medium was replaced with full medium and treated with 5 µg/ml trastuzumab or kept untreated. Corresponding to the FlexiTube GeneSolution, the AllStars Negative Control siRNA (5mol) and AllStars Negative Control siRNA AF488 (5 nmol) were used as negative controls. The AF488 labeling of the negative control allows analyzing the transfection efficiency through microscopic analysis with an appropriate filter set. Furthermore, the transfection efficiency was determined on a proteomic level

by western blot analysis. Therefore, samples were collected 24 hours after the transfection. In order to analyze the stability of the knockdown, additional samples were collected 120 hours after the transfection. This analyses were performed by M. Meyer.

2.2.6 Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics 23 & 24. The one-sample t-test was applied for analyzing normally distributed data against a fixed value. In this case, the untreated sample was set to 100 % with the treated samples from the same experiment referring to it. The two-sided Welch's t-test was applied using a pairwise comparison, testing variable data against each other. This analysis is only possible for data consisting of at least three independent datasets from three biologically independent experiments. For the statistical analysis of the speed in the motility analysis, a Mann-Whitney-U test was applied, due to the data not being normal distributed. Motility was analyzed using the chi-square test.

3 RESULTS

3.1 Cell line characterization

In this work, a panel of 12 gastric cancer cell lines was characterized.

In order to determine responder and non-responder cell lines to the treatment with trastuzumab different analyses were performed.

3.1.1 Proliferation

The proliferation was analyzed for all cell lines. Cells were treated with increasing concentrations of trastuzumab for 72 hours, measuring the metabolic activity as a surrogate marker for cell viability (Figure 10). This analysis was done by J. Kneissl (Kneissl et al. 2017). No significant changes in proliferation occurred for most of the cell lines. The cell lines GSU, H-111-TC and NCI-N87 responded to trastuzumab with a decrease in cell proliferation. The strongest decrease was detected for the cell line GSU, followed by H-111-TC. The cell line NCI-N87 showed only a small but significant decrease in proliferation. A concentration-dependent decrease in proliferation was observed for all cell lines. Higher concentrations than 10 µg/ml trastuzumab did not decrease the proliferation rates of the cell lines any further. For following analyses a concentration of 5 µg/ml trastuzumab was chosen. In Figure 10B, standard deviation is not indicated for better readability. Significant effects are shown in Table 7.

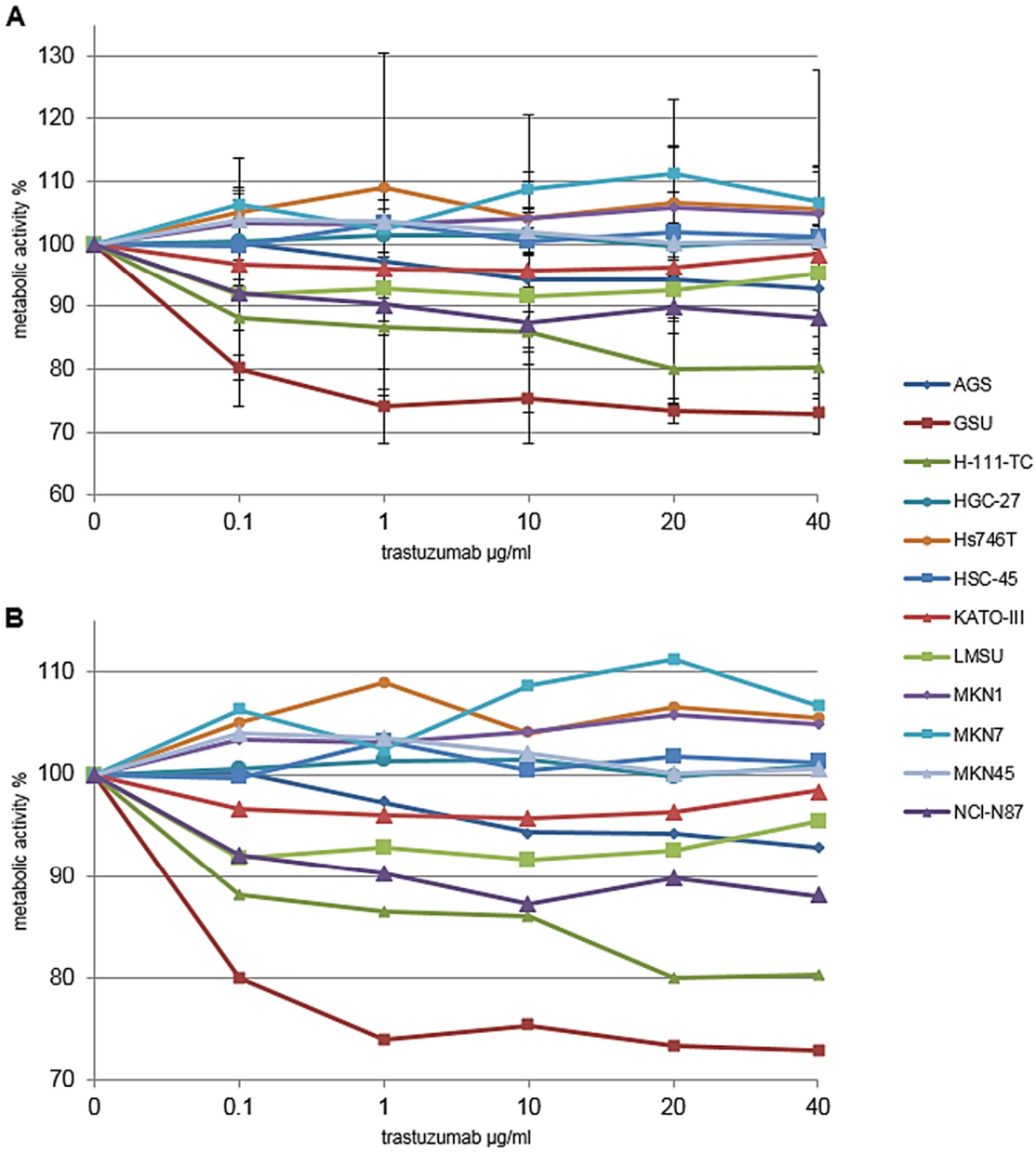


Figure 10: The effect of trastuzumab on cell proliferation of gastric cancer cell lines.
(A) For the analysis of the proliferation all cell lines were treated for 72 hours with trastuzumab. The metabolic activity was determined via WST-1 cell proliferation assay. Only GSU, H-111-TC and NCI-N87 cells showed a reduction in their proliferation under trastuzumab treatment. The mean value of three independent experiments is shown for each cell line. (B) The same analysis is displayed withouth standard deviations for better readability.

Table 7: Significant effects on the cell proliferation under trastuzumab treatment.
The effect of trastuzumab on proliferation was analyzed in the gastric cancer cell line panel. For each individual cell line, the untreated sample was set to 100 % to visualize the effect of the treatment. A statistical analysis (one-sample t-test) was performed, comparing the trastuzumab-treated samples with the untreated samples within one cell line. Significant effects were only obtained for the cell lines GSU, H-111-TC, MKN1, MKN7, MKN45 and NCI-N87. Significant effects are indicated by * 0.01 < p-value ≤ 0.05, ** 0.001 < p-value ≤ 0.01 or ***< 0.001 (one-sample t-test).

	cell line					
trastuzumab µg/ml	GSU	H-111-TC	MKN1	MKN7	MKN45	NCI-N87
0.1	0.009 / **	0.076	0.227	0.036 / *	0.229	0.005 / **
1	0.011 / *	0.161	0.047 / *	0.446	0.021 / *	0.075
10	0.004 / **	0.046 / *	0.037 / *	0.033 / *	0.460	0.007 / **
20	0.001 / ***	0.024 / *	0.054	0.048 / *	0.963	0.015 / *
40	0.002 / *	0.020	0.314	0.174	0.866	0.004 / **

3.1.2 Evaluation of different coatings for motility analysis

In an attempt to reveal differences in the phenotypic reaction to trastuzumab the motility of all cell lines of the gastric cancer cell line panel were planned to be analyzed. Before the motility analysis the best possible conditions for the cell lines to be motile needed to be determined. Therefore, cell culture dishes were coated with different coatings and concentrations of coatings. For collagen type I as a matrix, four different concentrations (50, 100, 130 and 150 µg/ml) were tested. Furthermore, fibronectin (10 µg/ml), laminin (1.65 µg/cm^2) and matrigel (4 mg/ml) were analyzed. The motility behavior of the cells concerning the coating was determined by eye and analyzed by time-lapse microscopy for some exemplary films (Figure 11). Motility was not observed for all cell lines. Only for the cell lines AGS, Hs746T, LMSU, MKN1, MKN7 and NCI-N87 motile cells were observed.

	Collagen type I								Fibronectin		Laminin		Matrigel	
	50 µg/ml		100 µg/ml		130 µg/ml		150 µg/ml		10 µg/ml		1. 65 µg/cm^2		4 mg/ml	
cell line	attachment	motility	attachment	motility	attachment	motility	attachment	motility	attachment	motility	attachment	motility	attachment	motility
AGS			+	+					-					
GSU	+	-	-	-	-		+	0	-				-	
H-111-TC	-		+	0	-		-		-				-	
HGC-27	-		+	0	-				-				-	
Hs746T			+	+										
HSC-45	-		+	0			+	-	-				-	
KATO-III			-										-	
LMSU			+	+					-					
MKN1			+	+										
MKN7	-		+	-			+	-	-		+	+	-	
MKN45	-		-		-									
NCI-N87	-		+	+									-	

Figure 11: Coating analysis for time-lapse microscopy
The coatings collagen type I, fibronectin, laminin and matrigel were analyzed for the gastric cancer cell line panel. First, the attachment of the cells to the coating was assessed. No attachment of the cells is indicated by -, whereas + indicated good attachment of the cells to the analyzed coating. The cell lines KATO-III and MKN45 did not attach to any coating. Bye eye the motility of the cells on the respective coatings was graduated into three levels; no motile cells (-), only few motile cells (0) and plenty of motile cells (+) observed.

3.1.3 Protein analysis

In order to determine the response to the treatment with trastuzumab on a molecular level, all cell lines were analyzed for protein expression and regulation via western blot analysis.

3.1.3.1 Western blot analysis

The direct effect of trastuzumab on HER2 activation was investigated addressing two different phosphorylation sites. For all cell lines untreated samples were generated and samples treated for 30 minutes with 5 µg/ml trastuzumab. The regulation of pHER2 was analyzed for the phosphorylation sites Y1221/1222 (Figure 12) and Y1248 (Figure 13).

For pHER2 Y1221/1222 no significant effects of trastuzumab were detectable in all cell lines, except for H-111-TC. An increase of the activation in trastuzumab-treated H-111-TC cells was observed. Because of various HER2 activation levels in the different cell lines the exposure time was adjusted for every cell line to ensure an adequate analysis. Thus, activation levels are not comparable between the different gastric cancer cell lines. For some cell lines, hardly any HER2 activation was detectable. Strong basal HER2 activation was observed for the cell lines AGS, GSU, MKN7 and NCI-N87.

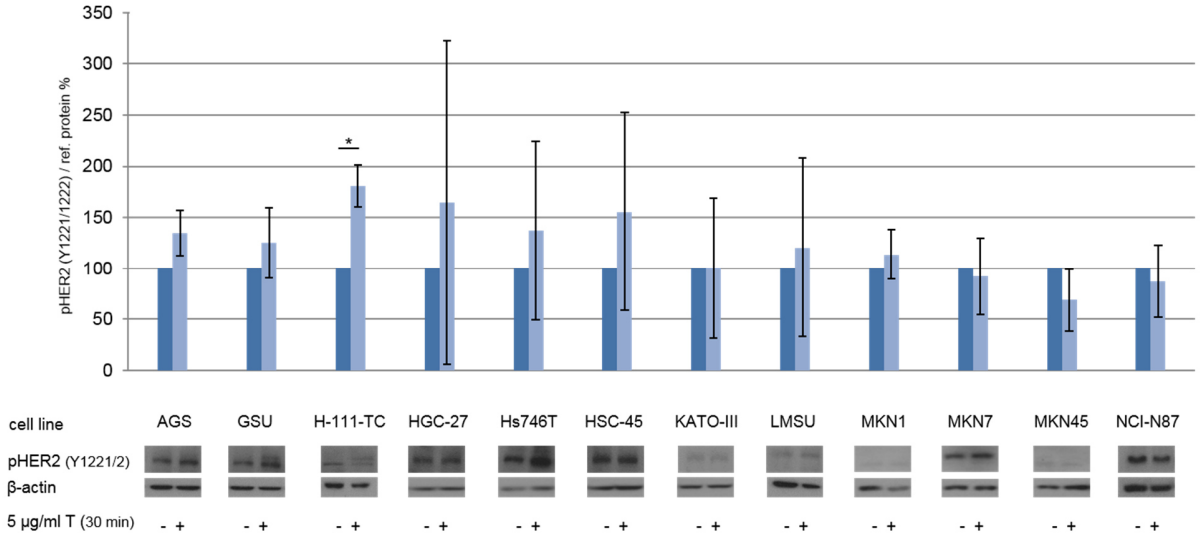


Figure 12: Effects of trastuzumab on the activation of HER2 (Y1221/1222) in the gastric cancer cell line panel.
Protein activation was detected in total lysates of untreated cells and cells treated with 5 µg/ml trastuzumab for 30 minutes by Western blotting using the phospho-specific antibody directed against pHER2 (Y1221/2). No significant decreasing effect of trastuzumab was detected, but an increase in the activation of HER2 in trastuzumab-treated H-111-TC cells. Quantitative signals are only comparable within one cell line. For each individual cell line, the untreated sample was set to 100 % to visualize the effect of the treatment. The mean values of three independent experiments are shown with one picture as an exemplary result. Significant effects are indicated by * $0.01 < \text{p-value} \leq 0.05$ (one-sample t-test).

For the analysis of pHER2 Y1248, a significant increase of HER2 activation was detected in trastuzumab-treated H-111-TC. A significant reduction of pHER2 was detected in the cell lines KATO-III and NCI-N87. For the remaining cell lines of the gastric cancer cell line panel no effect of trastuzumab on HER2 activation was detected. Exposure time was adjusted for each of the cell lines depending on the observed HER2 activation. Therefore, the results are not directly comparable between cell lines. For all cell lines HER2 activation was detectable with different intensities. Strong basal HER2 activation was observed for the cell lines Hs746T, MKN7 and NCI-N87.

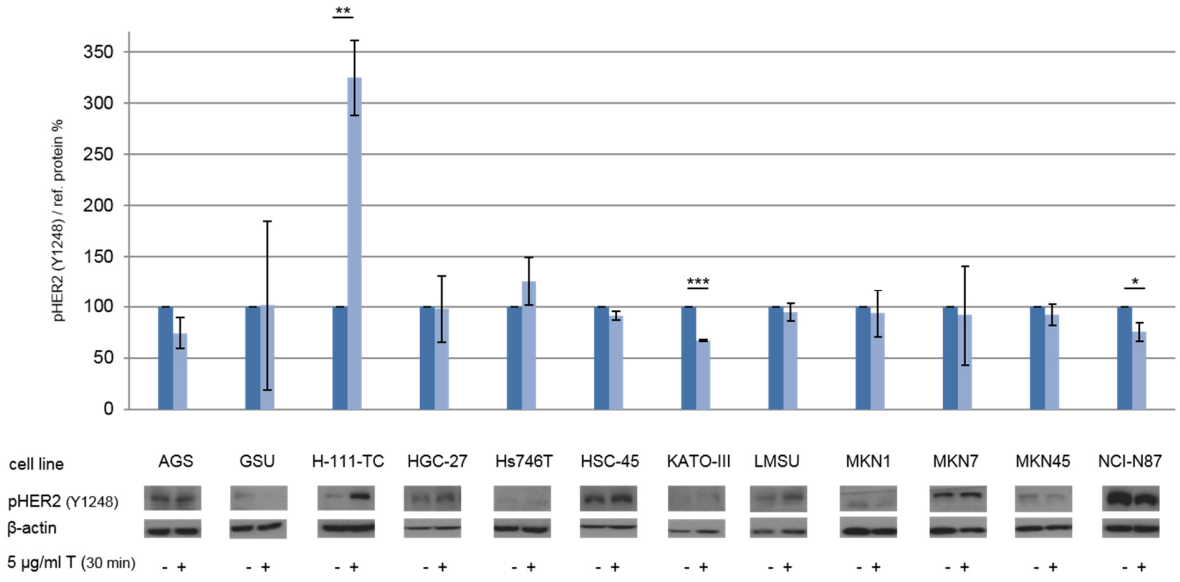


Figure 13: Effects of trastuzumab on the activation of HER2 (Y1248) in the gastric cancer cell line panel.
Protein activation was detected in total lysates of untreated cells and cells treated with 5 µg/ml trastuzumab for 30 minutes by Western blotting using the phospho-specific antibody directed against pHER2 (Y1248). A significant decreasing effect of trastuzumab was detected in the cell lines KATO-III and NCI-N87. An increase in the activation of HER2 was detected in trastuzumab-treated H-111-TC cells. Quantitative signals are only comparable within one cell line. For each individual cell line, the untreated sample was set to 100 % to visualize the effect of the treatment. The mean values of three independent experiments are shown with one picture as an exemplary result. Significant effects are indicated by * 0.01 < p-value ≤ 0.05, ** 0.001 < p-value ≤ 0.01 or ***< 0.001 (one-sample t-test).

The expression of HER2 was analyzed for all cell lines (Figure 14). The expression of HER2 was very different in the gastric cancer cell lines. Exposure time was adjusted for each of the cell lines depending on the observed HER2 expression. Therefore, the results are not directly comparable between cell lines. For all cell lines HER2 expression was detectable with different intensities. High HER2 expression was observed for the cell lines GSU, H-111-TC, MKN7 and NCI-N87. Only in KATO-III cells an effect of trastuzumab was visible, resulting in an increased HER2 expression.

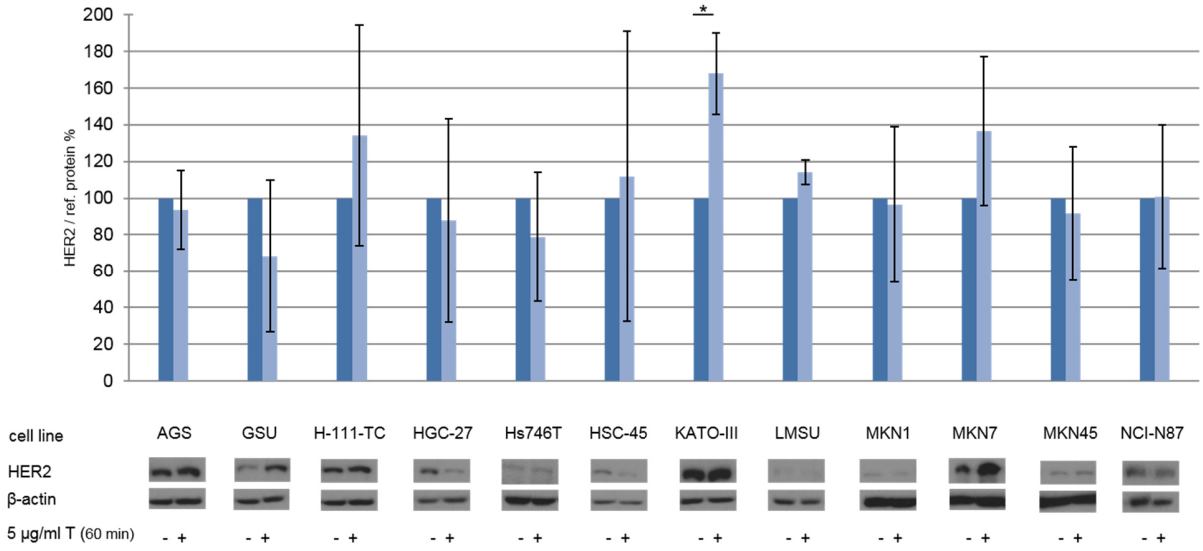


Figure 14: Effects of trastuzumab on the expression of HER2 in the gastric cancer cell line panel.
Protein expression was detected in total lysates of untreated cells and cells treated with 5 µg/ml trastuzumab for 60 minutes by Western blotting using a specific antibody directed against HER2. A significant increasing effect of trastuzumab was only detected in the cell line KATO-III. Quantitative signals are only comparable within one cell line. For each individual cell line, the untreated sample was set to 100 % to visualize the effect of the treatment. The mean values of three independent experiments are shown with one picture as an exemplary result. Significant effects are indicated by * 0.01 < p-value ≤ 0.05 (one-sample t-test).

EGFR is a possible dimerization partner of HER2. Therefore, the activation of EGFR was analyzed in all cell lines (Figure 15). Furthermore, the effect of trastuzumab on pEGFR was analyzed. Exposure time was adjusted for each of the cell lines depending on the observed EGFR activation. Therefore, the results are not directly comparable between cell lines. For all cell lines EGFR activation was detectable with different intensities. Protein activation was detected in total lysates of untreated cells and cells treated with 5 µg/ml trastuzumab for 60 minutes. No influence of trastuzumab on EGFR activation was detectable in the gastric cancer cell lines. Strong basal EGFR activation was observed for the cell lines Hs746T, KATO-III, MKN7 and MKN45. For the cell lines Hs746T and MKN45 two signals for pEGFR were detected.

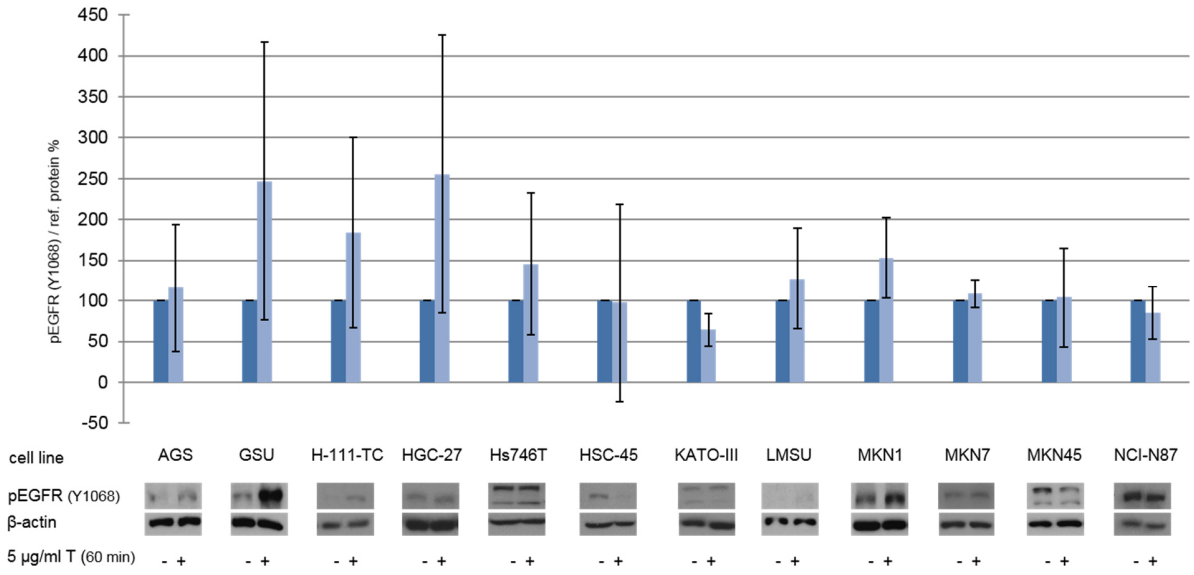


Figure 15: Effects of trastuzumab on the activation of EGFR in the gastric cancer cell line panel.
Protein activation was detected in total lysates of untreated cells and cells treated with 5 µg/ml trastuzumab for 60 minutes by Western blotting using the phospho-specific antibody directed against pEGFR (Y1068). No significant decreasing or increasing effect of trastuzumab was detected in the cell lines. Quantitative signals are only comparable within one cell line. For each individual cell line, the untreated sample was set to 100 % to visualize the effect of the treatment. The mean values of three independent experiments are shown with one picture as an exemplary result.

3.1.3.2 HER2 status of cell lines in FFPE-cell pellets

In addition to the determination of the HER2 expression by western blot analysis the HER2 status of all cell lines was determined by IHC and CISH as it is performed for gastric cancer patients. Both analyses were performed at the Institute of Pathology, Universitätsklinikum Leipzig. The cell lines were formalin-fixed, paraffin-embedded and H&E stained. Dr. med. G. Weirich reviewed the FFPE-cell pellets for suitability based on the H&E stainings. In non-suitable FFPE-cell pellets no cells were detected. FFPE-cell pellet samples classified as suitable showed high cell density and a sufficient amount of cells in the sections (Figure 16). This was mandatory to ensure an adequate staining of HER2 in the gastric cancer cell lines.

Figure 16: Example for a high-quality FFPE-cell pellet.
The sliced paraffin-embedded cell pellets were H&E stained and reviewed for suitability. The light pink color represents the HistoGel™ and the purple spots the cells. High cell density and a sufficient amount for both of the corresponding cell pellets was mandatory.

For all cell lines of the gastric cancer cell line panel an untreated FFPE-cell pellet was prepared. In addition, samples treated with trastuzumab were prepared for the cell lines MKN1, MKN7 and NCI-N87. A corresponding untreated cell pellet needed to be generated according to the different preparation protocol of the treated samples. Therefore, direct comparison was ensured. Similar, Hs746T and MKN1 cell samples were prepared treated with cetuximab and corresponding untreated samples. Both corresponding cell pellets were reviewed on their suitability for IHC and CISH. Samples classified as suitable showed high cell density and a sufficient amount of cells in the sections (Figure 17).

Figure 17: Example for high-quality FFPE-cell pellets of corresponding treated and untreated samples.
The sliced paraffin-embedded cell pellets were H&E stained and reviewed for suitability. The untreated cell pellet corresponds to the respective treated cell pellet. The light pink color represents the HistoGel™ and the purple spots the cells. High cell density and a sufficient amount for both of the corresponding cell pellets was mandatory.

The HER2 expression and amplification of the gastric cancer cell lines was analyzed in Leipzig by the UCCL (Table 8). All samples were sectioned and analyzed for HER2 by IHC and CISH. A. Monecke of the UCCL performed the scoring and determination of HER2 expression and amplification. Representative pictures of the HER2 status for each cell line are shown in Figure 18. The different scores, score +1 (10 % positively stained cells), score +2 (15 % positively stained cells) and score +3 (~100 % positively stained cells) were calculated for the analyzed samples. No HER2-positive cells were detected in the samples with score 0.

Three biological samples were analyzed resulting in small differences between the experiments. A score of 0 was observed for the cell lines AGS, HGC-27, Hs746T, KATO-III, LMSU, MKN1 and MKN45. A score of 1+ was determined for the cell line HSC-45. GSU and H-111-TC resulted in a score of 2+, whereas the cell lines MKN7 and NCI-N87 showed high HER2 expression with a score of 3+. The treatment of the different cells with trastuzumab or cetuximab did not influence the observed HER2 score in the cell lines (Table 9).

The CISH analysis was only performed for the untreated samples (Figure 18). A positive CISH-result was detected for H-111-TC, MKN7 and NCI-N87.

Table 8: IHC and CISH results for HER2 of the FFPE-cell pellets.
For each FFPE-cell pellet, an IHC- and CISH-score for HER2 was determined at the UCCL. Small differences occurred between the biological experiments. A. Monecke in Leipzig determined the score of the different cell lines for HER2. Score 3+ represents the highest possible score for HER2, thus every cell was stained positive for HER2. A score of 0 indicates no HER2-positive stained cells in the analyzed sample. Small percentages of positively stained cells in a sample were scored with score 1+ and score 2+.

cell line	treatment (time)	IHC-score	CISH
AGS	-	0	negative
GSU	-	2+	negative
H-111-TC	-	2+	positive
HGC-27	-	0	negative
Hs746T	-	0	negative
HSC-45	-	1+	negative
KATO-III	-	0	negative
LMSU	-	0	negative
MKN1		0	negative
MKN7	-	3+	positive
MKN45	-	0	negative
NCI-N87	-	3+	positive

Table 9: IHC score for HER2 for treated and corresponding untreated FFPE-cell pellets.
The cell lines NCI-N87, MKN7 and MKN1 were further treated with 5 µg/ml trastuzumab. Hs746T and MKN1 cells were treated with 1 µg/ml cetuximab. Due to different preparation steps for the treated FFPE-cell pellets compared to the untreated samples shown in Table 8, corresponding untreated cell pellets needed to be prepared. The treatment with trastuzumab or cetuximab did not influence the HER2 score.

cell line	treatment (time)	IHC-score
NCI-N87	5 µg/ml trastuzumab (4h)	3+
NCI-N87	untreated control for trastuzumab	3+
MKN7	5 µg/ml trastuzumab (4h)	3+
MKN7	untreated control for trastuzumab	3+
MKN1	5 µg/ml trastuzumab (4h)	0
MKN1	untreated control for trastuzumab	0
MKN1	1 µg/ml cetuximab (4h)	0
MKN1	untreated control for cetuximab	0
Hs746T	1 µg/ml cetuximab (4h)	0
Hs746T	untreated control for cetuximab	0

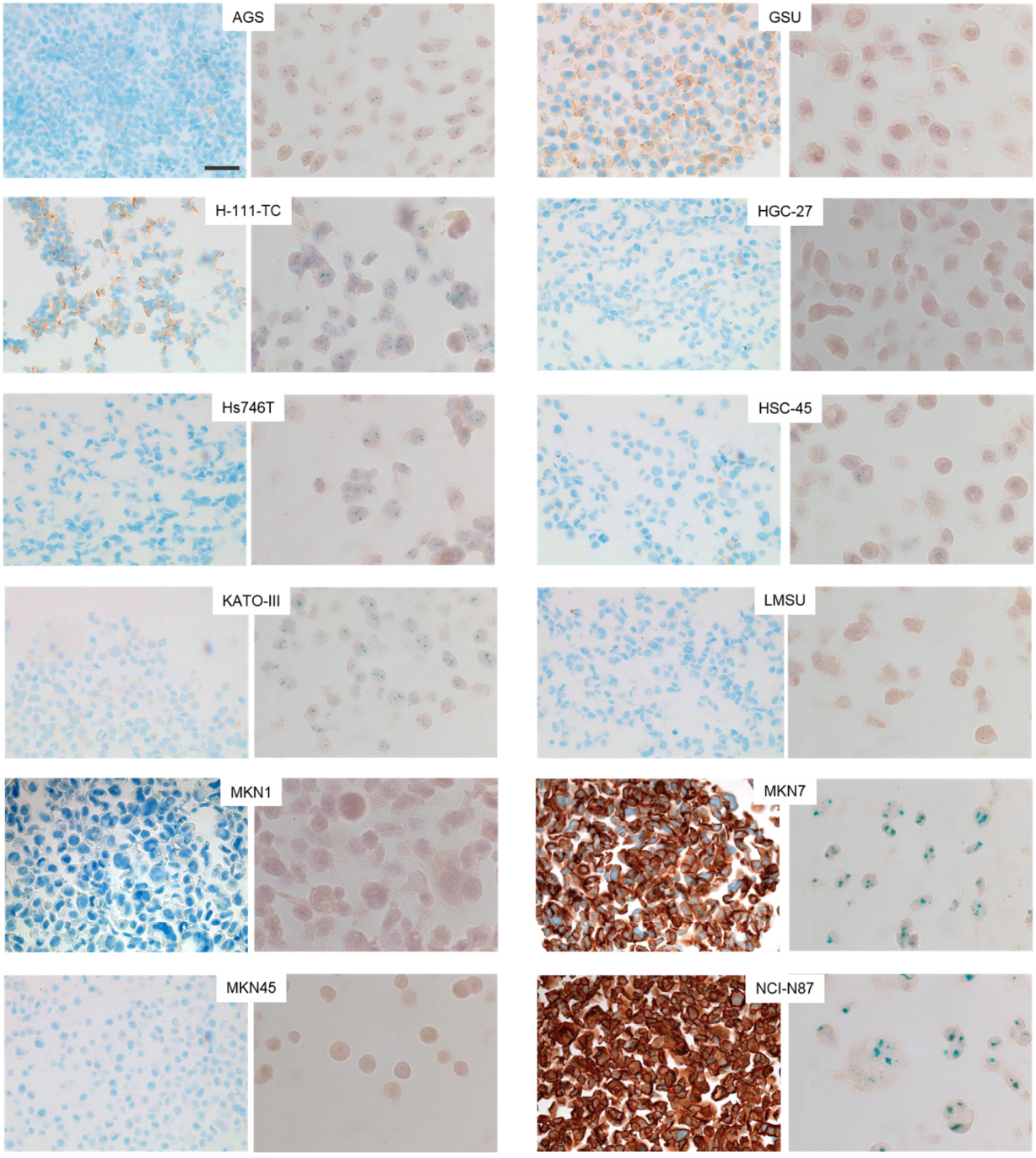


Figure 18: HER2 status of the gastric cancer cell lines.
For all gastric cancer cell lines a representative picture of the IHC and CISH for HER2 is shown. The size bar represents 50 µm and is representative for all analyzed cell lines. Representative for score 1+ and 2+, a slight staining of HER2 was detected by IHC for HSC-45, GSU and H-111-TC cells. For the cell lines MKN7 and NCI-N87 a score 3+ HER2 expression was observed. Via CISH, an amplification of *HER2* was detected in the cell lines H-111-TC, MKN7 and NCI-N87.

3.2 Analysis of responder and non-responder cell lines

The classification of the cell lines into responder and non-responder cell lines was based on the response to trastuzumab treatment in the proliferation analysis and the effects on HER2 activation. Moreover, the possibility of further motility analyses was required. The cell line NCI-N87 was determined as a responder cell line to the treatment with trastuzumab. Of the gastric cancer cell line panel the cell line NCI-N87 was the only cell line responding to trastuzumab in western blot and proliferation analysis. A reduced proliferation of NCI-N87 cells was observed under trastuzumab treatment in the proliferation analysis. In addition, trastuzumab treatment reduced the activation of HER2 in this cell line. For the cell lines MKN1 and MKN7, no response to trastuzumab in proliferation analysis was detected. Furthermore, trastuzumab treatment did not reduce HER2 activation in MKN1 and MKN7 cells. Ultimately crucial for the classification of these cell lines as non-responders was the possibility to examine these cell lines in the motility analysis, which was not possible for other possible non-responder candidates. Although low HER2 expression was observed for the cell line MKN1, this cell line was determined as non-responder cell line to the treatment with trastuzumab. The cell line MKN1 is a responder for cetuximab treatment, determined in an earlier project, thus an interesting cell line, responding differently to HER family directed antibodies. The cell line MKN7 was determined as a non-responder cell line to the treatment with trastuzumab. The cell line MKN7 harbors an *HER2* amplification as the cell line NCI-N87, therefore a direct comparison of the two cell lines is possible.
In the following a detailed molecular and phenotypic analysis of the three gastric cancer cell lines was performed.

3.2.1 Molecular analysis

All three cell lines were analyzed for their protein expression and activation by western blot and proteome profiler analysis. Moreover, the molecular effect of trastuzumab on the cell lines MKN1, MKN7 and NCI-N87 was evaluated.

3.2.1.1 Western Blot analysis

The cell lines MKN1, MKN7 and NCI-N87 were analyzed for HER2 expression and activation and the activation of EGFR, ERK and AKT in untreated samples and samples treated with 5 µg/ml trastuzumab.
To allow direct comparison of the HER2 expression and activation between the cell lines the signals were detected and analyzed together for all three cell lines. In Figure 19 the expression of

HER2 is shown. The lowest HER2 expression was detected for the cell line MKN1. MKN7 showed a strong signal for HER2 expression. Less protein was applied, because the expression of HER2 in NCI-N87 was too high to get comparable results. For the cell line NCI-N87 two signals for HER2 were detected. The HER2 expression was not influenced by trastuzumab treatment in any of the cell lines.

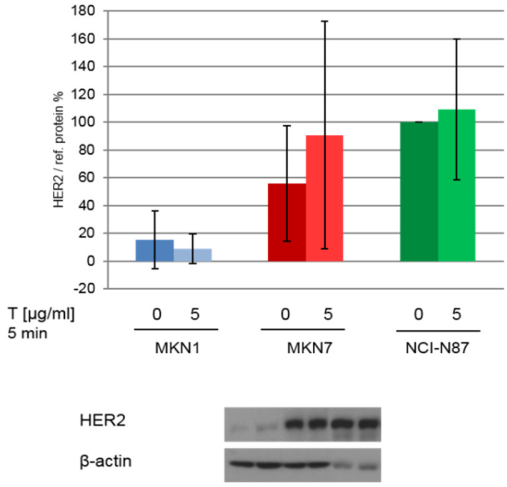


Figure 19: Direct comparison of HER2 expression for MKN1, MKN7 and NCI-N87.
Protein expression was detected in total lysates of untreated cells and cells treated with 5 µg/ml trastuzumab for 5 minutes by Western blotting using a specific antibody directed against HER2. No significant decreasing or increasing effect of trastuzumab was detected in the cell lines. The untreated sample of NCI-N87 was set to 100 % to visualize the effect of the treatment and to allow comparison between the cell lines. The highest expression of HER2 is detectable in the cell line NCI-N87. A slightly lower HER2 expression is detectable in MKN7 cells, whereas in MKN1 cells HER2 is hardly expressed. The mean values of three independent experiments are shown with one representative picture as an exemplary result.

Similar to the measurement of HER2 expression in the cell lines, the activation of HER2 was analyzed using the antibody pHER2 (Y1221/1222) (Figure 20). At the chosen exposure time to ensure direct comparison between the cell lines, hardly any HER2 activation was detectable for MKN1. The activation of HER2 in MKN7 was low, compared to the cell line NCI-N87, showing a strong HER2 activation. Less protein was applied for NCI-N87 to allow direct comparison between the cell lines. For the cell line NCI-N87 two signals for HER2 were detected. The activation of HER2 was not influenced by trastuzumab in MKN1 and MKN7. In NCI-N87 cells the activation of HER2 is slightly reduced under trastuzumab treatment.

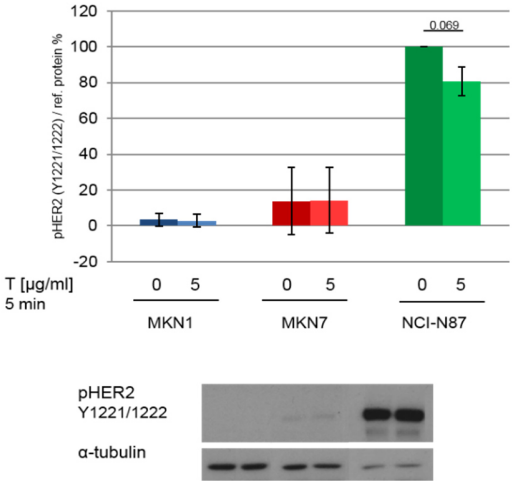


Figure 20: Direct comparison of HER2 activation for MKN1, MKN7 and NCI-N87.
Protein activation was detected in total lysates of untreated cells and cells treated with 5 µg/ml trastuzumab for 5 minutes by Western blotting using a phospho-specific antibody directed against pHER2 (Y1221/2). No significant decreasing or increasing effect of trastuzumab was detected in the cell lines. For NCI-N87 a small reduction of pHER2 was obtained. The untreated sample of NCI-N87 was set to 100 % to visualize the effect of the treatment and to allow comparison between the cell lines. The highest activation of HER2 is detectable in the cell line NCI-N87. Lower pHER2 levels are detectable in MKN7 cells, where in MKN1 cell HER2 is not activated. The mean values of four (three performed by K. Ebert) independent experiments are shown with one representative picture as an exemplary result.

The three cell lines were further investigated for HER2 activation independently (Figure 21). This was done to adjust the exposure time according to the signal intensities of the different cell lines. As previously observed in Figure 20, the activation of HER2 in MKN1 was very low and not influenced by trastuzumab treatment. MKN7 showed clear signals for pHER2 with no effect comparing the untreated with the trastuzumab-treated sample. The activation of HER2 was significantly reduced under trastuzumab treatment compared to the untreated sample in the cell line NCI-N87.

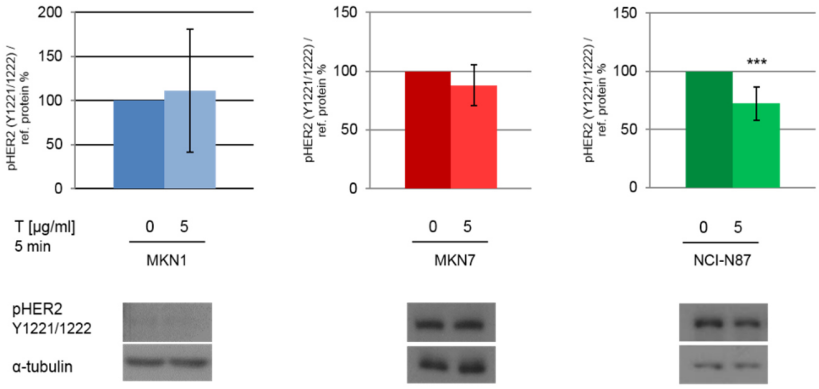


Figure 21: Activation of HER2 for the cell lines MKN1, MKN7 and NCI-N87.
Protein activation was detected in total lysates of untreated cells and cells treated with 5 µg/ml trastuzumab for 5 minutes by Western blotting using a phospho-specific antibody directed against pHER2 (Y1221/2). No significant decreasing or increasing effect of trastuzumab was detected in the cell lines MKN1 and MKN7. For NCI-N87 a significant reduction of HER2 activation was observed. The untreated samples were set to 100 % to visualize the effect of the treatment. Quantitative signals are only comparable within one cell line. The mean values of three independent experiments for MKN1, of six (two performed by S. Keller) independent experiments for MKN7 and of nine (four performed by S. Keller) independent experiments for NCI-N87 are shown with one representative picture as an exemplary result. Significant effects are indicated by p-value ***< 0.001 (one-sample t-test).

EGFR is a possible dimerization partner for HER2, thus indirectly influenced by the HER2-directed antibody trastuzumab. The activation of EGFR was analyzed in the cell lines MKN1, MKN7 and NCI-N87 using the antibody pEGFR (Y1068) (Figure 22). The measured levels for pEGFR were low for the cell line MKN1. MKN7 and NCI-N87 cells showed clear detectable signals for pEGFR. In none of the cell lines EGFR activation was influenced by trastuzumab treatment.

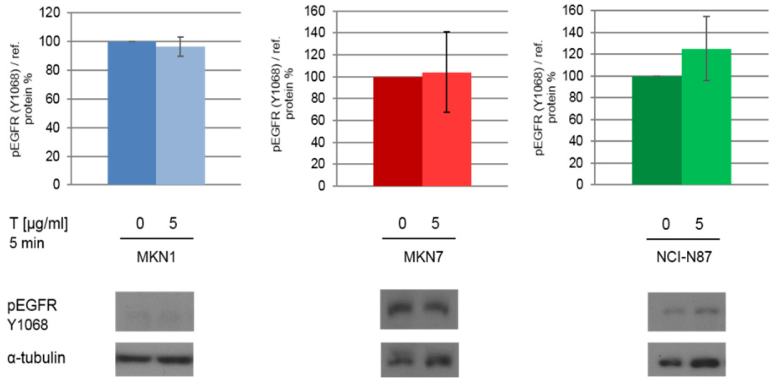


Figure 22: Activation of EGFR for the cell lines MKN1, MKN7 and NCI-N87.
Protein activation was detected in total lysates of untreated cells and cells treated with 5 µg/ml trastuzumab for 5 minutes by Western blotting using a phospho-specific antibody directed against pEGFR (Y1248). No significant decreasing or increasing effect of trastuzumab was detected in the cell lines. The untreated samples were set to 100 % to visualize the effect of the treatment. Quantitative signals are only comparable within one cell line. The mean values of three independent experiments for MKN1, of six (two performed by S. Keller) independent experiments for MKN7 and of five independent experiments for NCI-N87 are shown with one representative picture as an exemplary result.

The effect of trastuzumab on the downstream signaling was investigated by analyzing the activation of ERK (Figure 23) and the activation of AKT (Figure 24). For all cell lines ERK activation was detectable with similar signal intensities. For the cell line MKN1 a non-significant increase of ppERK was detected under trastuzumab treatment compared to the untreated sample. MKN7 did not respond to trastuzumab treatment. A strong increase in the activation of ERK was detected for the cell line NCI-N87.

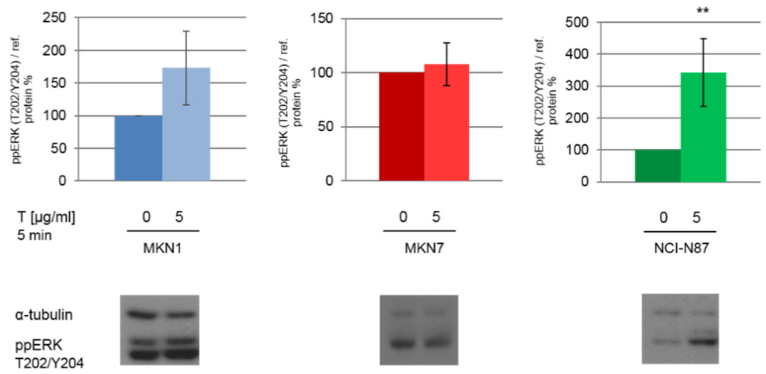


Figure 23: Activation of ERK for the cell lines MKN1, MKN7 and NCI-N87.
Protein activation was detected in total lysates of untreated cells and cells treated with 5 µg/ml trastuzumab for 5 minutes by Western blotting using a phospho-specific antibody directed against ppERK (T202/Y204). No significant decreasing or increasing effect of trastuzumab was detected in the cell lines MKN1 and MKN7. For NCI-N87 a significant induction of ppERK was observed. The untreated samples were set to 100 % to visualize the effect of the treatment. Quantitative signals are only comparable within one cell line. The mean values of three independent experiments for MKN1, of four independent experiments for MKN7 and of five independent experiments for NCI-N87 are shown with one representative picture as an exemplary result. Significant effects are indicated by ** 0.001 < p-value ≤ 0.01 (one-sample t-test).

The activation of AKT was clearly detectable in all cell lines. Trastuzumab did not influence the activation of AKT.

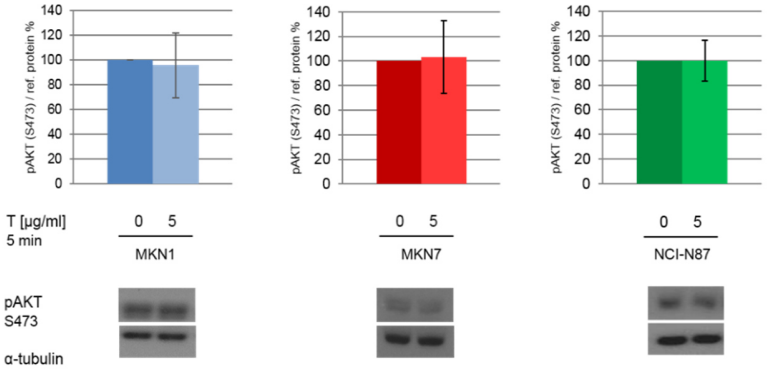


Figure 24: Activation of AKT for the cell lines MKN1, MKN7 and NCI-N87.
Protein activation was detected in total lysates of untreated cells and cells treated with 5 µg/ml trastuzumab for 5 minutes by Western blotting using a phospho-specific antibody directed against pAKT (S473). No significant decreasing or increasing effect of trastuzumab was detected in the cell lines. The untreated samples were set to 100 % to visualize the effect of the treatment. Quantitative signals are only comparable within one cell line. The mean values of three independent experiments for MKN1, of four independent experiments for MKN7 and NCI-N87 are shown with one representative picture as an exemplary result.

The cell lines were further investigated in a kinetic analysis in order to reveal time dependent effects of trastuzumab. Untreated samples were prepared at time points corresponding to the time points of motility, invasion and proliferation analysis. Samples treated with trastuzumab for short and long time periods were prepared and analyzed.

For the cell line MKN1 the signals for pHER2 were hardly detectable, therefore no reliable analysis of HER2 activation was possible (Suppl. Figure: 1). For AKT activation a culture time dependent effect was observed. The activation of AKT was reduced over time in the untreated samples 22

hours and 72 hours compared to the untreated sample prepared at time point 0 hours (Suppl. Figure: 1). An increase of AKT activation was detected for trastuzumab treated samples (4 h and 7 h) compared to untreated samples 22 hours and 72 hours, but not when compared to the corresponding untreated samples 0 hours and 7 hours.

The activation of pEGFR was not significantly altered in MKN1 (Suppl. Figure: 2). For the activation of ERK only a culture time depending effect in the untreated sample 72 h was detected (Suppl. Figure: 2).

In the kinetic analysis of MKN7 cells no effect was detectable for the activation of HER2 and AKT (Suppl. Figure: 3). Signals for pHER2 were hardly measureable.

In the cell line MKN7 the analysis of pEGFR resulted in small differences comparing different trastuzumab treatment times. Comparing the untreated sample 72 hours with a treatment of 10 minutes with trastuzumab a significant increase of pEGFR was detected (Suppl. Figure: 4). The activation of ERK was strongly influenced by trastuzumab in the cell line MKN7 (Suppl. Figure: 4). Comparing the untreated sample 0 hours with different treatment times, the samples treated for 30 minutes and 72 hours with trastuzumab resulted in a decrease of ppERK. The activation of ERK was significantly reduced comparing the sample treated for 72 hours with the untreated sample prepared after 7 hours. Similar effects were obtained for the sample 30 minutes being compared with untreated 72 hours. Further, significant effects were detected comparing samples to each other, which were treated with trastuzumab for different time points.

HER2 activation was reduced under trastuzumab treatment after 10 minutes, 4 hours, 22 hours and 72 hours compared to the untreated sample prepared at 0 hours in the cell line NCI-N87 (Suppl. Figure: 5). Comparing the trastuzumab treated samples 22 hours and 72 hours to the corresponding untreated samples (22 hours and 72 hours), no significant effect of trastuzumab was observed. Further, effects were detected comparing samples to each other, which were treated with trastuzumab for different time points. The activation of AKT was influenced by trastuzumab at 7 and 72 hours compared to the untreated samples prepared at 0 hours (Suppl. Figure: 5). All other effects were detected comparing trastuzumab-treated samples at different time points to each other.

For the activation of EGFR strong effects were detected (Suppl. Figure: 6). A culture time depending effect occurred comparing the untreated sample 0 hours with the untreated sample 7 hours. Furthermore, trastuzumab dependent effects were detected for treatment durations of 30 minutes, 1 hour, 4 hours and 72 hours compared to untreated 0 hours. The effect in the samples 1 hour and 4 hours also occurred by comparing pEGFR with the untreated samples prepared after 7 hours. The effects detected for pERK were diverse (Suppl. Figure: 6). Mainly trastuzumab

depending effects occurred comparing different treatment times to each other. An increase of ppERK in the 10 minutes trastuzumab-treatment sample, and a decrease after 72 hours of trastuzumab was detected compared to the untreated sample 0 hours, and when compared to 7 hours untreated. Furthermore, significant effects were detected by comparing the untreated sample 22 hours with trastuzumab treatment (5 min, 10 min, 30 min and 4 h).

For the trastuzumab responder cell line NCI-N87 further proteins were analyzed on their activation and the influence of trastuzumab (Figure 25). HER2 activation was analyzed addressing the phosphorylation site Y1248. A significantly reduced HER2 activation was observed under trastuzumab treatment. In order to identify crosstalk between HER2 and other RTKs, the activation of HER3 and MET was analyzed under trastuzumab treatment. For the activation of HER3 and MET no significant effects of trastuzumab were detected in NCI-N87 cells.

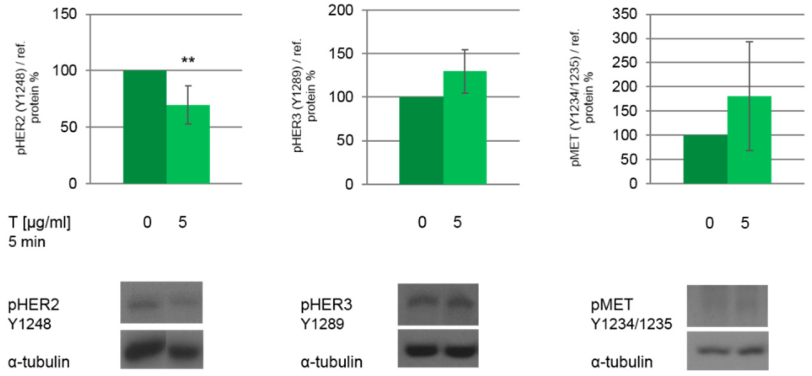


Figure 25: Effect of trastuzumab on pHER2 (Y1248), pHER3 and pMET in NCI-N87.
For the trastuzumab responder cell line NCI-N87 the activation of further proteins was analyzed. Protein activation was detected in total lysates of untreated cells and cells treated with 5 µg/ml trastuzumab for 5 minutes by Western blotting using phospho-specific antibodies directed against pHER2 (Y1248), pHER3 (Y1289) and pMET (Y1234/1235). The activation of pHER2 (Y1248) was reduced under trastuzumab treatment. No significant effects of trastuzumab were detected for pHER3 and pMET. The untreated samples were set to 100 % to visualize the effect of the treatment. The mean values of six independent experiments for pHER2 and four independent experiments for pHER3 and pMET are shown with one picture as an exemplary result. Significant effects are indicated by ** $0.001 < \text{p-value} \leq 0.01$ (one-sample t-test).

3.2.1.2 Proteome Profiler

The specific antibodies used for western blot analysis detect only one phosphorylation site, whereas pan-antibodies used in proteome profiler analyses detect several phosphorylation sites. Thus to analyze the three cell lines on a broader spectrum a Proteome Profiler Phospho-RTK array, detecting the tyrosine phosphorylation of 49 human RTKs and a Kinase Proteome Profiler array for 43 kinase phosphorylation sites were performed. Only small effects of trastuzumab on a molecular level analyzed by western blot analysis were detected. Due to these small effects the

tyrosine kinase inhibitor afatinib was added as an internal control treatment. All results have been published by Keller, Zwingenberger and colleagues (Keller et al. 2018).

3.2.1.2.1 RTK Proteome Profiler

For each of the analyzed cell lines different proteins were identified to be activated and regulated under treatment.

MKN1 cells showed activated AXL, EGFR, Eph B2, Eph B3, IGF1-R, Insulin R, RYK and Tie-2. Remarkably, ROR2 was activated in the cell line MKN1 but not in the cell line NCI-N87. Under treatment, the activation of EGFR changed exclusively (Figure 26). Trastuzumab treatment had no effect in the cell line MKN1. The activation of EGFR was increased by EGF and non-significantly decreased by afatinib treatment.

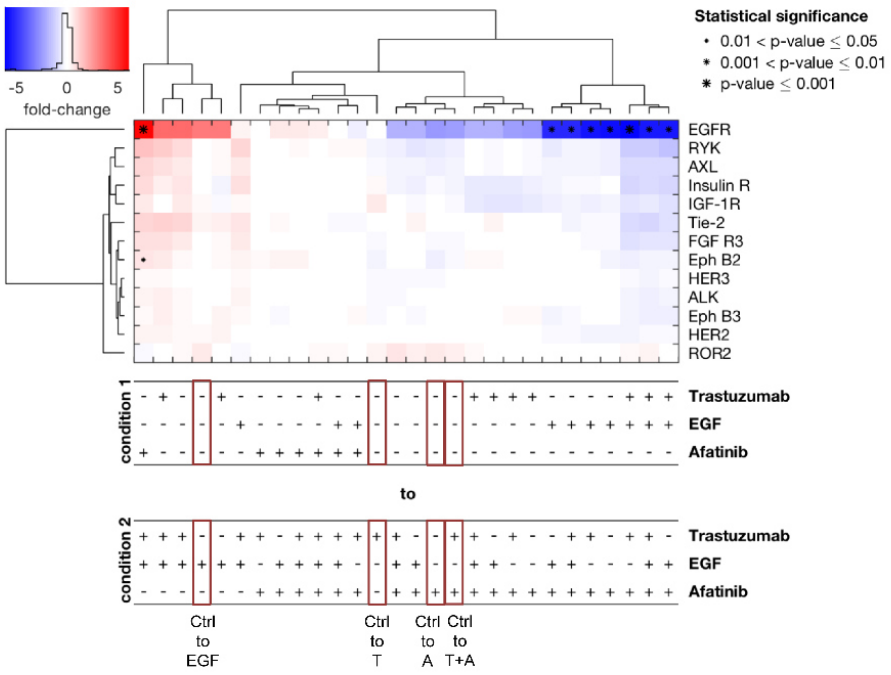


Figure 26: RTK Proteome Profiler analyzed by a mixed-effect model with clustering and statistical analysis for the cell line MKN1.

The RTK Proteome Profiler was performed to detect the effects of trastuzumab, afatinib and/or EGF treatment on different RTKs in MKN1 cells. The cells were treated for 20 min with 5 µg/ml trastuzumab, 0.5 µM afatinib or 5 ng/ml EGF as well as in combination with each other. The phosphorylation levels were quantified using densitometrical analysis; a linear mixture modelling was inferred. In the cluster analysis, the x-fold change of each activated protein is shown. Samples were clustered due to the similarity of the activated proteins and treatment conditions. The experiment was performed three times. Significant effects between different treatment conditions are indicated by (*) with increasing size (0.01 < p-value ≤ 0.05, 0.001 < p-value ≤ 0.01 and p-value ≤ 0.001) (Keller et al. 2018).

The cell line MKN7 showed activated EGFR, HER2, HER3, IGF-1R, Insulin R and RYK. The strong activation of AXL and, as observed in the cell line MKN1, ROR2 was visible. All treatments almost exclusively changed the activation of EGFR and HER2 (Figure 27). Trastuzumab treatment resulted in a small, non-significant decrease of HER2 phosphorylation. Afatinib slightly, non-significantly decreased the activation of EGFR and HER2. The combination of afatinib and trastuzumab showed a similar inhibition as afatinib mono-treatment. Under treatment with EGF, the activation of EGFR was increased. The difference in the inhibition of EGFR between afatinib-treated cells and EGF-treated cells was more pronounced than between afatinib-treated and non-treated cells.

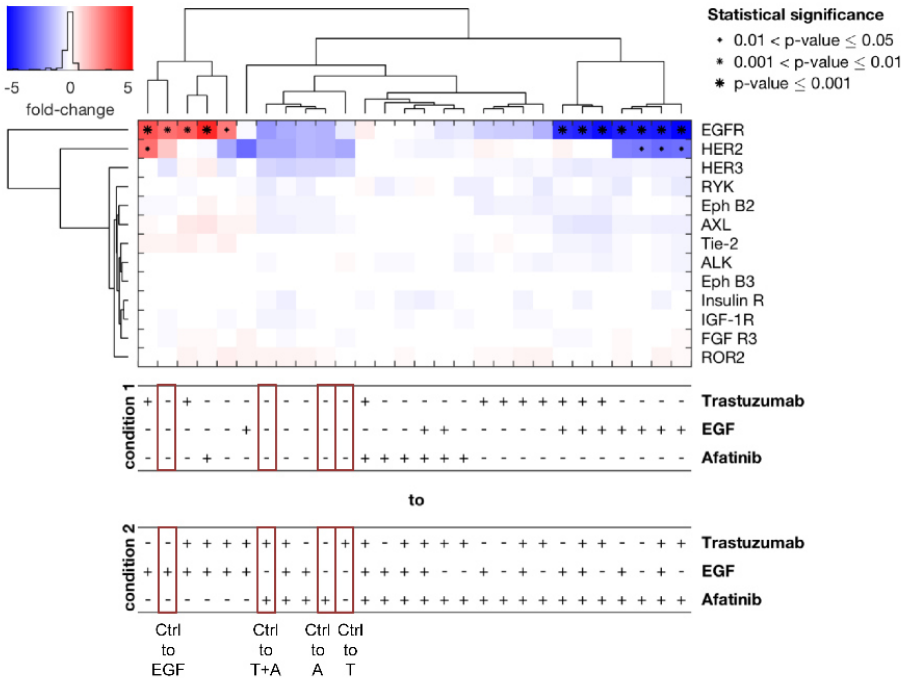


Figure 27: RTK Proteome Profiler analyzed by a mixed-effect model with clustering and statistical analysis for the cell line MKN7.
The RTK Proteome Profiler was performed to detect the effects of trastuzumab, afatinib and/or EGF treatment on different RTKs in MKN7 cells. The cells were treated for 20 min with 5 µg/ml trastuzumab, 0.5 µM afatinib or 5 ng/ml EGF as well as in combination with each other. The phosphorylation levels were quantified using densitometrical analysis; a linear mixture modelling was inferred. In the cluster analysis, the x-fold change of each activated protein is shown. Samples were clustered due to the similarity of the activated proteins and treatment conditions. The experiment was performed three times. Significant effects between different treatment conditions are indicated by (*) with increasing size (0.01 < p-value ≤ 0.05, 0.001 < p-value ≤ 0.01 and p-value ≤ 0.001) (Keller et al. 2018).

In the cell line NCI-N87, the activation of ALK, EGFR, HER2, HER3, FGF R3, IGF-1R, Insulin R and RYK was detected in untreated cells. Trastuzumab treatment resulted in a reduction of pHER2, pEGFR and a slight, non-significant decrease of pHER3 in compared to the untreated

sample (Figure 28). Cells treated with afatinib showed a decrease of pEGFR, pHER3 and a slight, non-significant decrease of pHER2 compared to not treated cells. The combination of trastuzumab plus afatinib resulted in a strong decrease of pEGFR, pHER2 and pHER3. Consequently, an additional effect of trastuzumab in combination with afatinib was observed on pHER2 in comparison to afatinib mono-treatment. EGFR phosphorylation was increased after treatment with EGF. The decrease of pEGFR under trastuzumab plus/or afatinib was more prominent in the EGF-treated cells compared to the untreated cells.

The clustering with respect to treatment conditions revealed the strong impact of the compounds EGF and afatinib. The analysis indicated that the inhibiting effect of afatinib treatment was consistent across all conditions in all cell lines, while the effect of trastuzumab was visible in the lower levels of the tree. This shows that the effect was context-specific and independent of the condition.

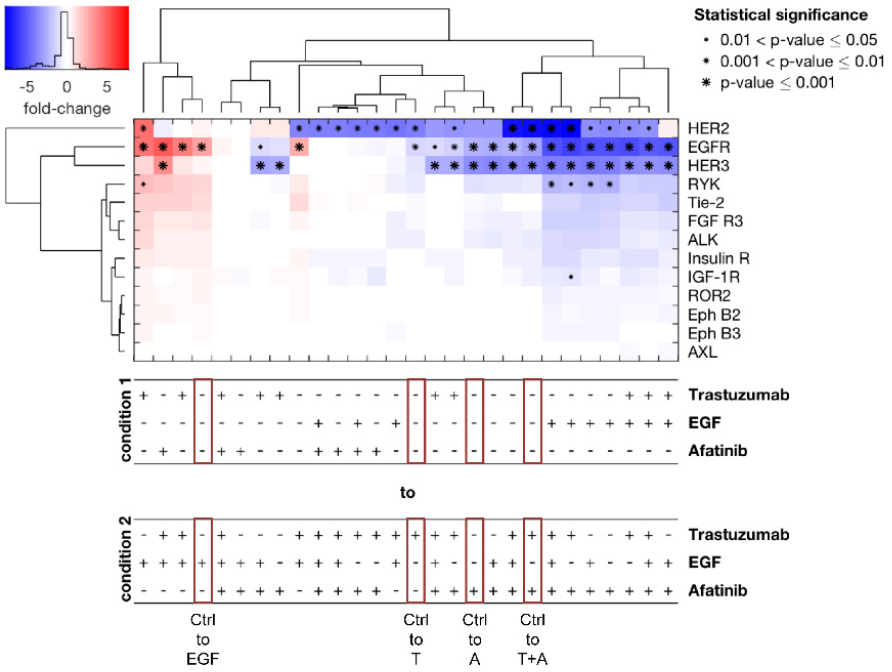


Figure 28: RTK Proteome Profiler analyzed by a mixed-effect model with clustering and statistical analysis for the cell line NCI-N87.
The RTK Proteome Profiler was performed to detect the effects of trastuzumab, afatinib and/or EGF treatment on different RTKs in NCI-N87 cells. The cells were treated for 20 min with 5 µg/ml trastuzumab, 0.5 µM afatinib or 5 ng/ml EGF as well as in combination with each other. The phosphorylation levels were quantified using densitometrical analysis; a linear mixture modelling was inferred. In the cluster analysis, the x-fold change of each activated protein is shown. Samples were clustered due to the similarity of the activated proteins and treatment conditions. The experiment was performed three times. Significant effects between different treatment conditions are indicated by (*) with increasing size (0.01 < p-value ≤ 0.05, 0.001 < p-value ≤ 0.01 and p-value ≤ 0.001) (Keller et al. 2018).

3.2.1.2.2 Kinase Proteome Profiler

All of the kinases analyzed in the Kinase Proteome Profiler were active in the cell lines MKN1, MKN7 and NCI-N87. The analyses of kinase activation in MKN1, MKN7 and NCI-N87 cells was done by S. Keller.

In the cell line MKN1, trastuzumab treatment did not result in a quantifiable alteration of the activation state of the kinases, regardless of mono- or combination treatment (Figure 29). The protein activations of ERK1/2, CREB, p70 S6 kinase, EGFR and FAK were strongly influenced by EGF and afatinib treatment. The activation of ERK1/2 was induced by EGF and NRG1 treatment. The treatment of MKN1 cells with afatinib or in combination plus trastuzumab resulted in inhibition of ERK1/2 activation. EGF treatment but not NRG1 treatment was able to activate CREB and p70 S6 kinase compared to the untreated cells. EGFR and FAK activation was significantly increased by EGF. EGFR did not cluster with any of the other kinases.

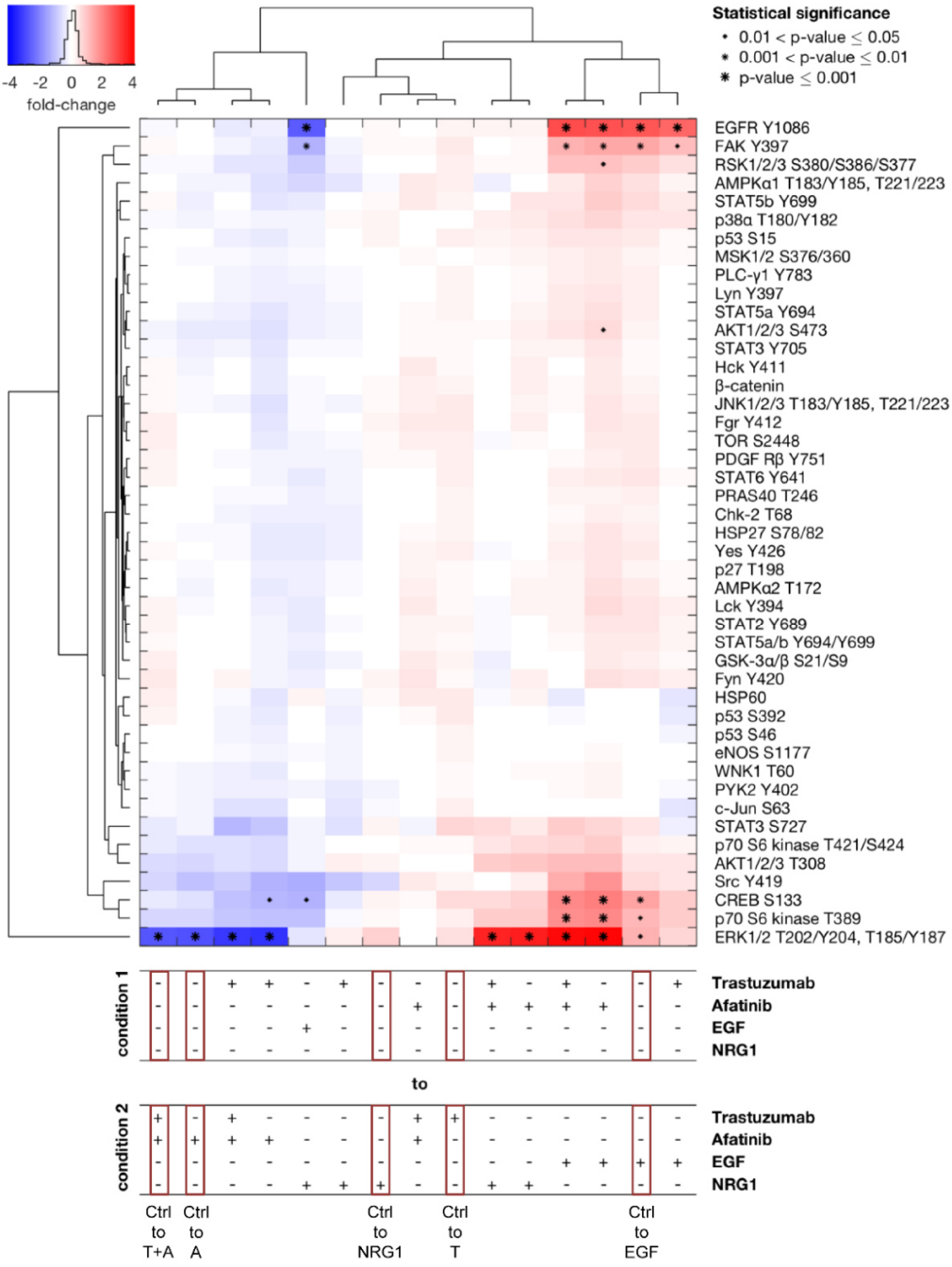


Figure 29: Effects of inhibition and activation of receptors on the phosphorylation of a panel of kinases in MKN1 cells. The Kinase Proteome Profiler was performed to detect the effects of trastuzumab and/or afatinib treatment on downstream kinases in MKN1 cells. The cells were treated for 20 min with 5 µg/ml trastuzumab, 0.5 µM afatinib or the combination of both drugs or were stimulated for 5 min with 5 ng/ml EGF or 20 ng/ml NRG1. The phosphorylation levels were quantified using densitometrical analysis; a linear mixture modelling was inferred. In the cluster analysis, the x-fold change of the activation of each included protein is shown. Samples were clustered due to the similarity of the activated proteins and treatment conditions. The experiment was performed three times. Significant effects between different treatment conditions are indicated by (*) with increasing size (0.01 < p-value ≤ 0.05, 0.001 < p-value ≤ 0.01 and p-value ≤ 0.001) (Keller et al. 2018).

Trastuzumab showed no measurable effect on the analyzed kinases in MKN7 cells (Figure 30). Furthermore, the combination of trastuzumab plus afatinib resulted in a similar reduction of activated kinases when compared to afatinib mono-treatment. The activation of ERK1/2, AKT1/2/3, WNK1 and EGFR was strongly influenced by the different treatments. After EGF and NRG1 treatment, the activation of ERK1/2, AKT1/2/3 and WNK1 was unchanged compared to untreated cells. Afatinib treatment resulted in a reduced activation of ERK1/2, AKT1/2/3 and WNK1. EGFR appears to be not significant phosphorylated by EGF treatment. In the cluster analysis, EGFR phosphorylation clustered completely different from the detectable downstream kinases.

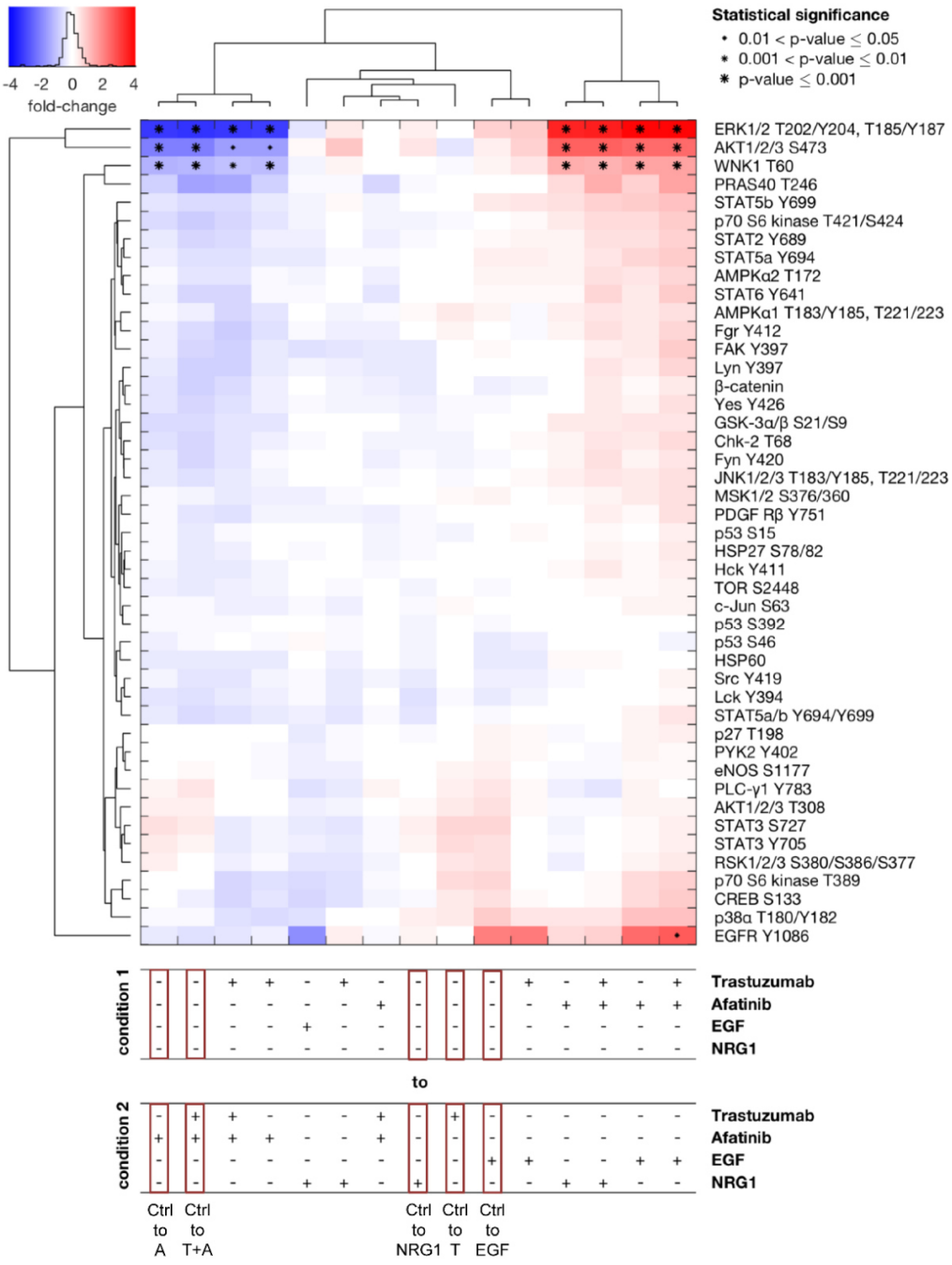


Figure 30: Effects of inhibition and activation of receptors on the phosphorylation of a panel of kinases in MKN7 cells.
The Kinase Proteome Profiler was performed to detect the effects of trastuzumab and/or afatinib treatment on downstream kinases in MKN7 cells. The cells were treated for 20 min with 5 µg/ml trastuzumab, 0.5 µM afatinib or the combination of both drugs or were stimulated for 5 min with 5 ng/ml EGF or 20 ng/ml NRG1. The phosphorylation levels were quantified using densitometrical analysis; a linear mixture modelling was inferred. In the cluster analysis, the x-fold change of the activation of each included protein is shown. Samples were clustered due to the similarity of the activated proteins and treatment conditions. The experiment was performed three times. Significant effects between different treatment conditions are indicated by (*) with increasing size (0.01 < p-value ≤ 0.05, 0.001 < p-value ≤ 0.01 and p-value ≤ 0.001) (Keller et al. 2018).

In the cell line NCI-N87, the phosphorylation profile of kinases was similar for trastuzumab-treated and untreated cells (Figure 31). Moreover, cell treated with trastuzumab plus afatinib showed the same activation profile as cells treated only with afatinib. Consequently, trastuzumab had no measurable influence on the activation of downstream kinases and the analyzed receptors EGFR and PDGF Rβ. In contrast, a decreased EGFR phosphorylation was detectable for NCI-N87 in the RTK proteome profiler array. This inconsistency can be explained by pan-antibodies used in the RTK array and phosphorylation site-specific antibodies in the kinase array. Under the stimulation of NCI-N87 cells with EGF and the inhibition of HER receptors by afatinib, few proteins were strongly regulated. These proteins were EGFR, ERK1/2, AKT1/2/3, PRAS40, STAT5b and WNK1. EGF treatment resulted in stronger EGFR activation compared to untreated cells. This effect was also observed for the comparison of EGF with the other treatments. Treatment of NCI-N87 cells with EGF or NRG1 resulted in a small, non-significant increase or no increase at all of pERK1/2, pAKT1/2/3, pPRAS40 and pWNK1 compared to untreated cells. A reduction of AKT1/2/3, PRAS40, WNK1 and non-significant for ERK1/2 phosphorylation was observed in afatinib-treated cells compared to untreated cells. Remarkably, EGFR did not cluster with any of the downstream kinases.

The analysis across conditions revealed that the effect of afatinib treatment was consistent across conditions while the effect of trastuzumab treatment was context-specific.

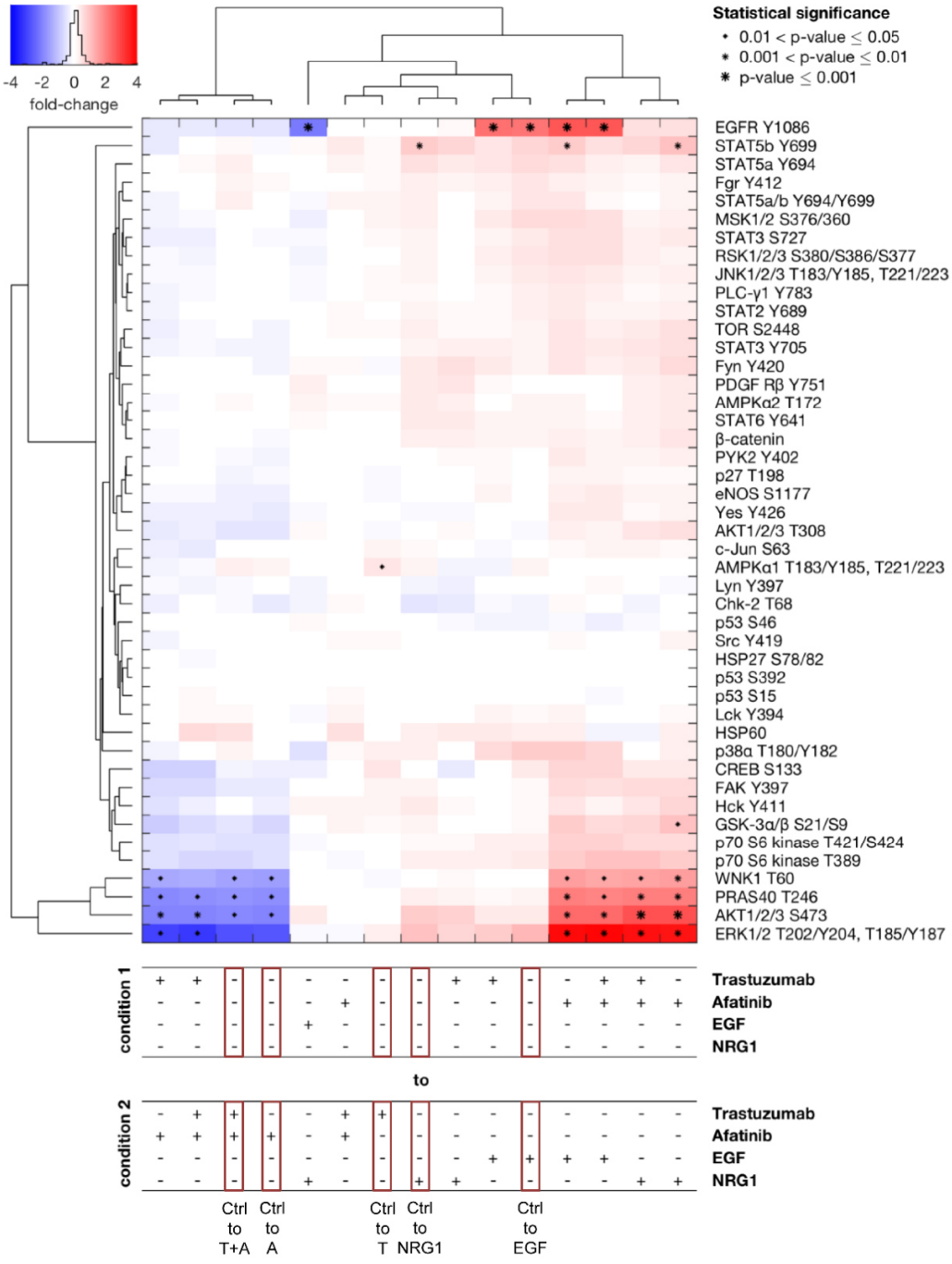


Figure 31: Effects of inhibition and activation of receptors on the phosphorylation of a panel of kinases in NCI-N87 cells. The Kinase Proteome Profiler was performed to detect the effects of trastuzumab and/or afatinib treatment on downstream kinases in NCI-N87 cells. The cells were treated for 20 min with 5 µg/ml trastuzumab, 0.5 µM afatinib or the combination of both drugs or were stimulated for 5 min with 5 ng/ml EGF or 20 ng/ml NRG1. The phosphorylation levels were quantified using densitometrical analysis; a linear mixture modelling was inferred. In the cluster analysis, the x-fold change of the activation of each included protein is shown. Samples were clustered due to the similarity of the activated proteins and treatment conditions. The experiment was performed three times. Significant effects between different treatment conditions are indicated by (*) with increasing size (0.01 < p-value ≤ 0.05, 0.001 < p-value ≤ 0.01 and p-value ≤ 0.001) (Keller et al. 2018).

3.2.2 Phenotypic analysis

3.2.2.1 Motility

All cell lines have been analyzed earlier for suitable coatings for motility analysis. Further motility analyses were performed for the cell lines MKN1, MKN7 and NCI-N87. Suitable coatings for these three cell lines are presented in Table 10. MKN1 and NCI-N87 cells were analyzed by S. Keller and me, while MKN7 cells were analyzed by K. Ebert. Motility analysis of MKN1 and NCI-N87 was performed on 100 µg/ml collagen type I as matrix with a pre-cell culture time of 1 hour. For the cell line MKN7 a coating of 1.65 µg/cm^2 laminin was applied. The cells needed to be pre-cultured for 24 hours to ensure attachment of the cells.

Table 10: Suitable coatings and pre-cell culture time for the cell lines MKN1, MKN7 and NCI-N87.
Different coatings were analyzed for the trastuzumab responder and non-responder cell lines. For MKN1 and NCI-N87 a coating of 100 µg/ml collagen type I and a pre-cell culture time of 1 h was most appropriate. The cell line MKN7 needed to be pre-cultured for 24 h because of slow attachment to the cells. A coating of 1.65 µg/cm^2 laminin was the most suitable for this cell line.

cell line	coating	pre-cell culture time
MKN1	collagen type I 100 µg/ml	1 h
MKN7	laminin 1.65 µg/cm^2	24 h
NCI-N87	collagen type I 100 µg/ml	1 h

Time-lapse films were analyzed semi-automatically. With this evaluation a determination of motile cells was possible (Figure 32). Cells were determined as motile, when leaving their area, covered at the beginning, completely during the film. The “percentage of motile cells” was similar in untreated MKN1 and untreated MKN7 cells. The “percentage of motile cells” was elevated in untreated NCI-N87 cells compared to the other two cell lines. No significant impact of trastuzumab treatment on the “percentage of motile cells” was detected for MKN1 cells. For MKN7 and NCI-N87 cells a small, not-significant decrease of motile cells was detected in the samples being treated with trastuzumab.

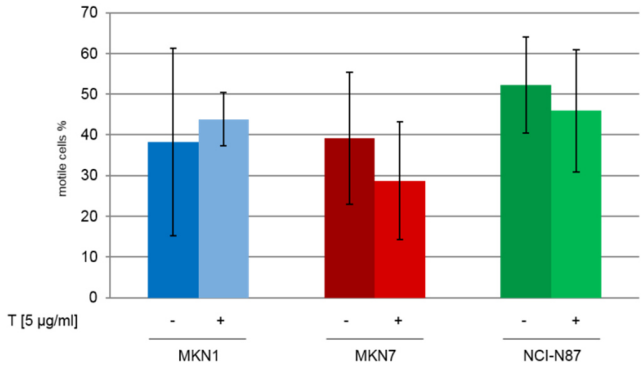


Figure 32: Cell motility of MKN1, MKN7 and NCI-N87 cells.
Motility was analyzed in untreated cells and cells treated for 7 hours with 5 µg/ml trastuzumab. Each cell in the analysis was traced throughout the film. Cells were determined as motile, when leaving their area, covered at the beginning, completely during the film. Trastuzumab treatment did not show significant effects in MKN7 and NCI-N87 cells, but a small decrease in motile cells was visible under treatment for both cell lines. The mean values of three independent experiments are shown.

Furthermore, speed was calculated for all time-lapse microscopy films (Figure 33). Speed was determined as the distance of a cell, divided by the film length. The detected speed in MKN1 cells was not influenced by trastuzumab treatment. MKN7 cells showed slightly reduced speed comparing the untreated samples to MKN1 cells. Trastuzumab resulted in a small not-significant decrease of speed in the cell line MKN7. For NCI-N87 no influence of trastuzumab was detected on cell speed. Comparing the untreated samples of MKN1, MKN7 and NCI-N87, a basal lower speed was detected for NCI-N87 cells.

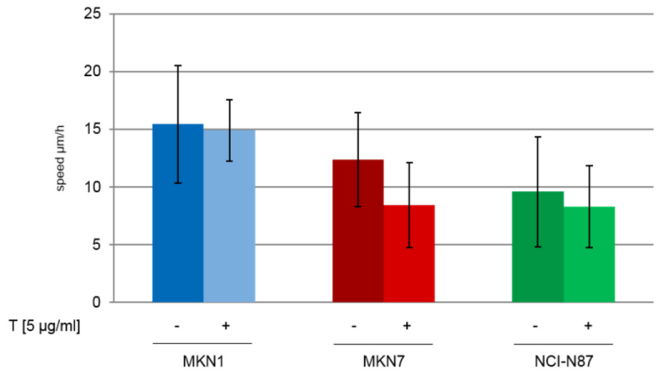


Figure 33: Calculated speed of MKN1, MKN7 and NCI-N87 cells.
The speed of untreated cells and cells treated for 7 hours with 5 µg/ml trastuzumab was analyzed. Speed was determined as the distance of a cell, divided by the film length. No significant effects occurred. Reduced speed was visible in the samples treated trastuzumab in MKN7 and NCI-N87 cells. The mean values of three independent experiments are shown.

3.2.2.2 Proliferation

In Figure 34 the proliferation analysis is shown in detail only for the cell lines MKN1, MKN7 and NCI-N87. The data is based on the proliferation analysis performed by J. Kneissl, shown in Figure 10. For the cell lines MKN1 and MKN7 no decreasing proliferation under trastuzumab treatment was detected. A slight increase of metabolic activity was observed for both cell lines in the trastuzumab-treated samples. The cell line NCI-N87 revealed a reduction of proliferation under trastuzumab treatment. The proliferation of NCI-N87 cells was reduced to 90 % under trastuzumab treatment. The different applied concentrations of trastuzumab did not result in a strong influence on the metabolic activity.

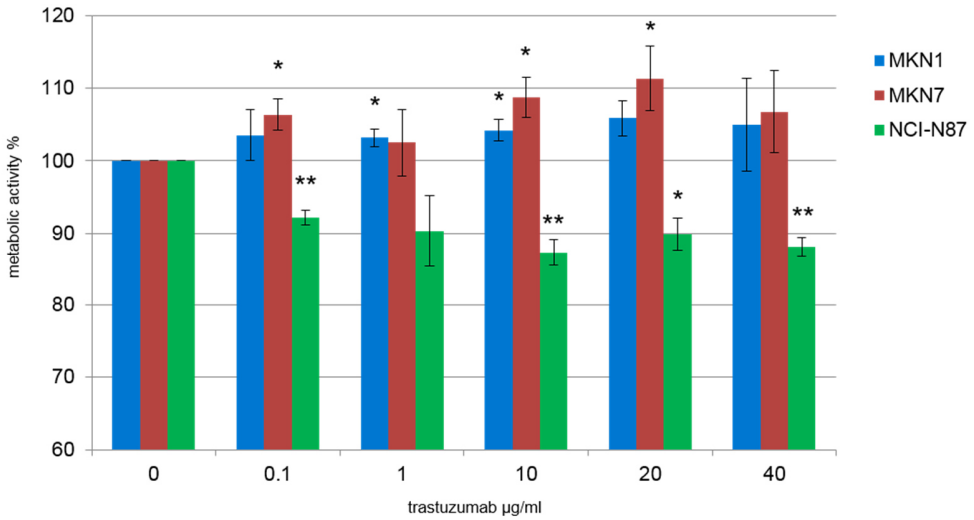


Figure 34: Effect of trastuzumab on the cell proliferation of MKN1, MKN7 and NCI-N87 cells.
To analyze the effect of trastuzumab on cell proliferation, the cell lines were treated for 72 h with trastuzumab. The metabolic activity was determined via WST-1 cell proliferation assay. The cell line NCI-N87 was trastuzumab sensitive, showing a decrease in proliferation. The proliferation of the cell lines MKN1 and MKN7 was not reduced under trastuzumab treatment. The mean values of three independent experiments are shown. Significant effects compared to untreated control cells are indicated by * 0.01 < p-value ≤ 0.05 or ** 0.001 < p-value ≤ 0.01 (one-sample t-test).

3.2.2.3 Invasion

Furthermore, the invasiveness of the three gastric cancer cell lines MKN1, MKN7 and NCI-N87 was analyzed (Figure 35). A commercial invasion assay was applied. The cells were treated for 22 hours with 5 µg/ml trastuzumab, untreated cells serving as a control. The invasiveness is not comparable between the gastric cancer cell lines. Trastuzumab treatment increased the invasiveness of MKN1 cells. For the cell line MKN7 no effect of trastuzumab on the invasiveness was observed. The invasiveness of NCI-N87 cells was significantly reduced under trastuzumab treatment.

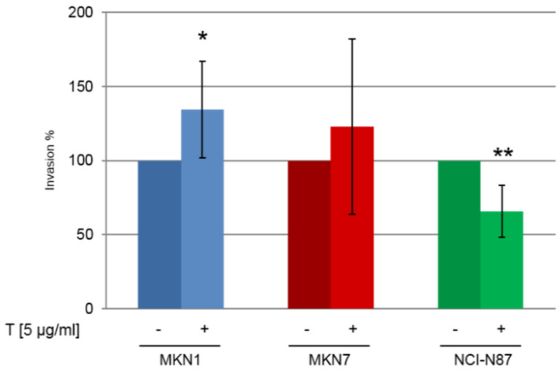


Figure 35: Effects of trastuzumab on the invasiveness in MKN1, MKN7 and NCI-N87.
The invasiveness of untreated cells and cells treated for 22 hours with 5 µg/ml trastuzumab was analyzed. An increasing effect of trastuzumab was detected in the cell line MKN1. The invasiveness of NCI-N87 was decreased under trastuzumab treatment. For each individual cell line, the untreated sample was set to 100 % to visualize the effect of the treatment. The mean values of six independent experiments are shown for MKN1 and MKN7. For NCI-N87, seven independent experiments are presented. Significant effects are indicated by * 0.01 < p-value ≤ 0.05 or ** 0.001 < p-value ≤ 0.01 (one-sample t-test).

3.3 Validation of possible resistance factors

3.3.1 HER2 shedding

HER2 shedding is a known resistance factor in breast cancer (Colomer et al. 2000, Leitzel et al. 1995) and was suspected to be associated with the response to trastuzumab in the cell lines MKN1, MKN7 and NCI-N87. Therefore, the HER2 shedding was analyzed by detecting the intracellular and the extracellular domain of HER2 in all three cell lines. Samples were generated for the four previously described untreated time points (0 h, 7 h, 22 h and 72 h). Moreover, cells treated with 5 µg/ml trastuzumab and 10 µg/ml trastuzumab were analyzed. The possible effect of pervanadate on HER2 shedding as positive control was investigated in two further samples. Codony-Servat et al reported the cleavage of the HER2 ectodomain to be a pervanadate-activable process (Codony-Servat et al. 1999).

3.3.1.1 Intracellular HER2 domain

The HER2 ICD was investigated using an antibody detecting all forms of membrane-bound HER2 (results not shown). For MKN1 only the full-length HER2 receptor was observed. Nevertheless, small treatment effects on total HER2 expression occurred. A decrease of HER2 expression was detected in trastuzumab treated samples (5 µg/ml T: 5 min, 72 h; 10 µg/ml T: 72 h) compared with the untreated sample prepared at 0 hours. In the cell line MKN7 mainly the full-length HER2 receptor was detected with small fractions of the shedded forms. Significant differences occurred comparing treated samples to each other. A significant reduction of HER2 expression was observed for the untreated sample 0 hours compared to the sample treated for 72 hours with 5 µg/ml trastuzumab. The analysis of the cell line NCI-N87 showed all forms of HER2 being detectable. The effect of trastuzumab on total HER2 expression was visible by analyzing all HER2 forms. Different treatment time points with 5 µg/ml trastuzumab resulted in a reduced HER2 expression compared to the untreated sample 0 hours. A culture time-dependent significant reduction of HER2 expression was further observed comparing the untreated sample 0 hours with the sample untreated 72 hours.

3.3.1.2 Extracellular HER2 domain

The HER2 ECD was analyzed performing a commercial ELISA kit, detecting only the extracellular domain of HER2 (Figure 36). Samples were prepared simultaneously to the samples for the analysis of the HER2 ICD.

In order to compare the results of three biological experiments within one cell line and between cell lines, E. Raimundez performed a batch correction and a scaling.

The values of HER2 ECD were below the detection limit of the assay for MKN1 cells. In the cell line MKN7 HER2 ECD was mainly influenced by cell culture time with no influence of any treatment. A strong increase of HER2 ECD levels was observed over time. For the cell line NCI-N87 the highest HER2 ECD levels were detected. Comparing the treatment effects with the appropriate untreated controls a small, but not significant effect was observed. Furthermore, a small impact of the culture time is detectable.

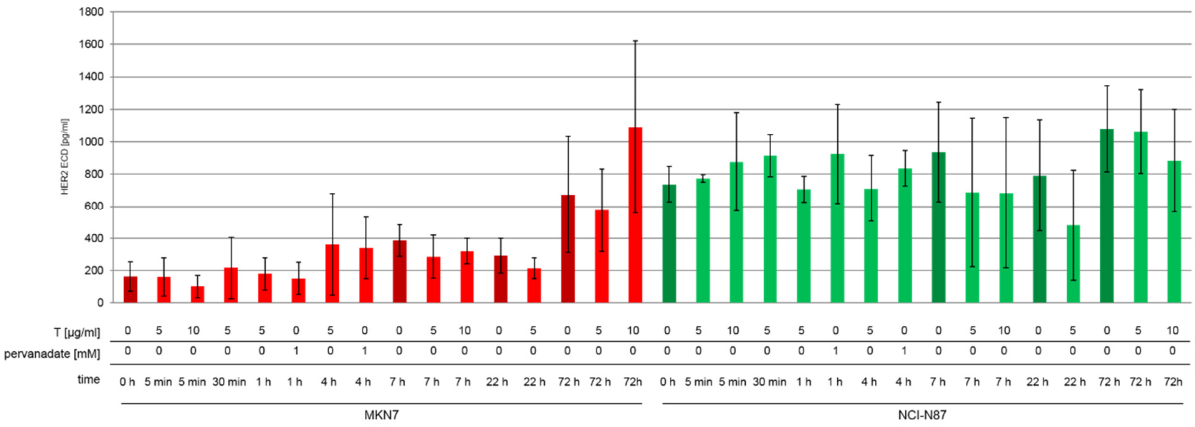


Figure 36: HER2 ECD measurement.
The shedded extracellular part of HER2 was detected by ELISA. Samples were treated with 5 µg/ml trastuzumab, 10 µg/ml trastuzumab or 1 mM pervanadate for different time points. For MKN1 the levels of HER2 ECD were below detection limit. The cell line MKN7 showed increasing HER2 ECD levels with increasing cell culture time, not influenced by any treatment. HER2 ECD levels for NCI-N87 were slightly influenced by trastuzumab treatment with no cell culture time-dependent changes. For direct comparison, the data of the three biological experiments were batch corrected and a scaling was done.

3.3.2 Analysis of AXL

In the RTK Proteome Profiler a strong activation of AXL was detected in the cell line MKN7 but not in the trastuzumab responder cell line NCI-N87. An overexpression of AXL is described in many carcinomas (Brown et al. 2016) and associated with a negative prognosis in breast cancer (Liu et al. 2009). Furthermore, resistances to different therapies due to AXL are known (Mahadevan et al. 2007). Therefore, AXL expression was suspected to play a role in the response to trastuzumab in the cell line MKN7. The analysis of AXL in MKN7 cells was performed by M. Meyer.

3.3.2.1 Establishment of the siRNA knockdown

To establish a knockdown of AXL in MKN7 cells different concentrations of siRNA were analyzed. As controls, cells were furthermore treated with negative-control siRNA, a negative-control siRNA

with an attached fluorescence or lipofectamine only. Samples were prepared after 24 hours. To ensure a stable knockdown for the following proliferation assay the knockdown was analyzed further after 120 hours.

For a concentration of 10 pmol a knockdown of AXL to ~20 % was detected (Figure 37). A clear reduction of the signal in western blot analysis was visible. The knockdown of AXL was stable for 120 hours. No significant effects occurred in the other samples.

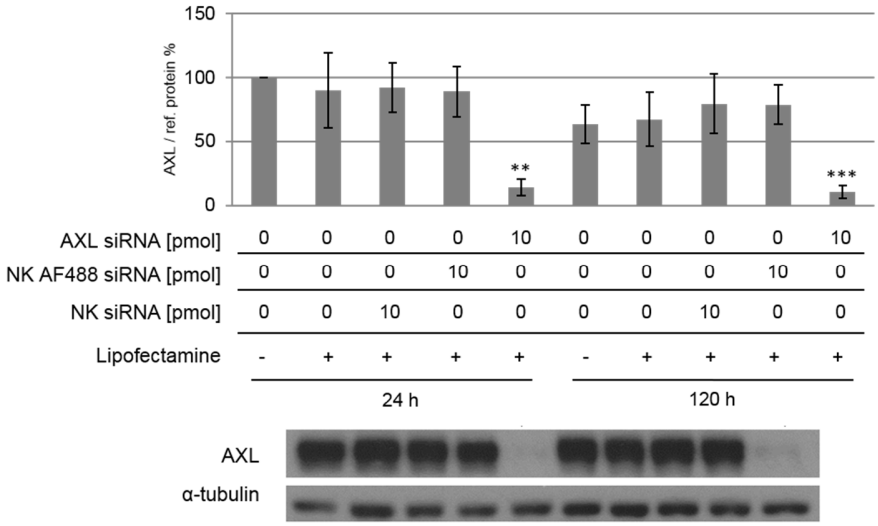


Figure 37: Establishment of the AXL siRNA transfection with a concentration of 10 pmol in MKN7.
The effect of 10 pmol AXL siRNA and three negative controls was analyzed in MKN7 cells. The negative controls (NK siRNA, NK AF488 siRNA and lipofectamine only) did not influence the expression of AXL. Under the treatment of AXL siRNA the expression was reduced. Furthermore, the effects were stable up to 120 hours. The untreated samples were set to 100 % to visualize the effect of the different treatments. The mean values of three independent experiments are shown with one picture as an exemplary result. Significant effects are indicated by ** 0.001 < p-value ≤ 0.01 or ***< 0.001 (one-sample t-test).

Further concentrations of siRNA were analyzed. A concentration of 50 pmol resulted in an effective knockdown (Figure 38). The expression of AXL was reduced to ~15 % after 24 hours and 120 hours. No significant effect occurred in the other samples.

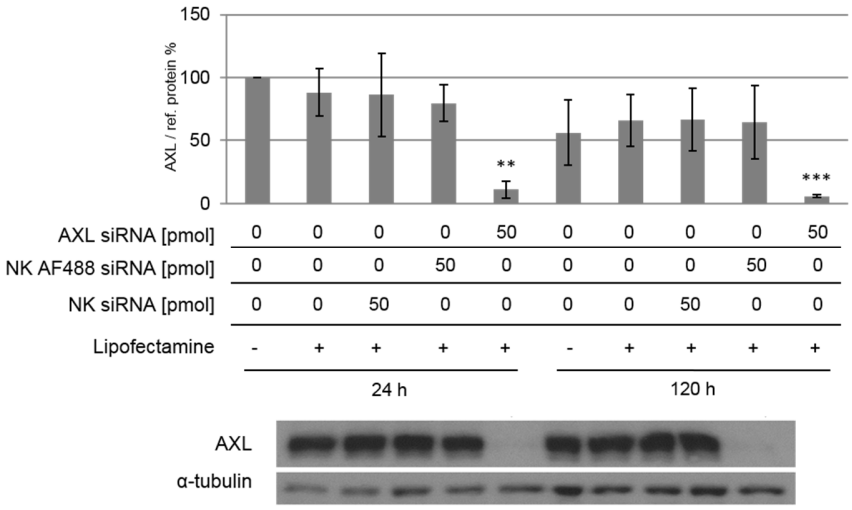


Figure 38: Establishment of the AXL siRNA transfection with a concentration of 50 pmol in MKN7.
The effect of 50 pmol AXL siRNA and three negative controls was analyzed in MKN7 cells. The negative controls (NK siRNA, NK AF488 siRNA and lipofectamine only) did not influence the expression of AXL. Under the treatment of AXL siRNA the expression was reduced. Furthermore, the effects were stable up to 120 hours. The untreated samples were set to 100 % to visualize the effect of the different treatments. The mean values of three independent experiments are shown with one picture as an exemplary result. Significant effects are indicated by ** 0.001 < p-value ≤ 0.01 or ***< 0.001 (one-sample t-test).

Another concentration of 76 pmol AXL siRNA resulted in unspecific effects in addition to the effective knockdown of AXL (Figure 39). The siRNA reduced the expression of AXL to ~ 15 %. The knockdown of AXL was stable for 120 hours.

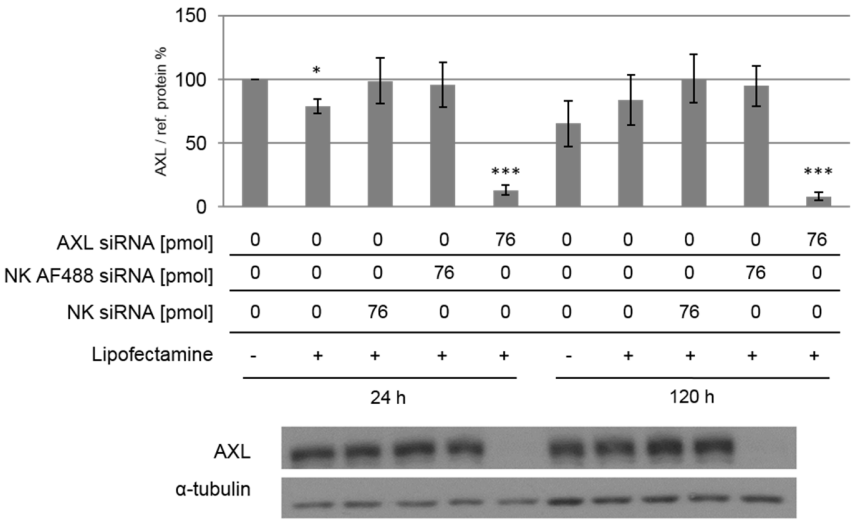


Figure 39: Establishment of the AXL siRNA transfection with a concentration of 76 pmol in MKN7.
The effect of 76 pmol AXL siRNA and three negative controls was analyzed in MKN7 cells. The negative controls (NK siRNA, NK AF488 siRNA) did not influence the expression of AXL. Lipofectamine showed a small effect in the analyzed samples. Under the treatment of AXL siRNA the expression was reduced. Furthermore, the effects were stable up to 120 hours. The untreated samples were set to 100 % to visualize the effect of the different treatments. The mean values of three independent experiments are shown with one picture as an exemplary result. Significant effects are indicated by * 0.01 < p-value ≤ 0.05 or ***< 0.001 (one-sample t-test).

For further analyses a concentration of 50 pmol AXL siRNA was chosen.

3.3.2.2 Analysis of the effect on trastuzumab response

3.3.2.2.1 Proliferation

The proliferation was investigated in the differently treated MKN7 cells (Figure 40). Untreated cells, NK siRNA and AXL siRNA transfected cells were analyzed. Cells were treated with increasing concentrations of trastuzumab, measuring the metabolic activity as a surrogate marker for cell viability. A small decrease in the metabolic activity was visible under trastuzumab, but no significant change in proliferation occurred in all samples. The metabolic activity was slightly elevated in the AXL siRNA samples compared to untreated and negative-control siRNA cells.

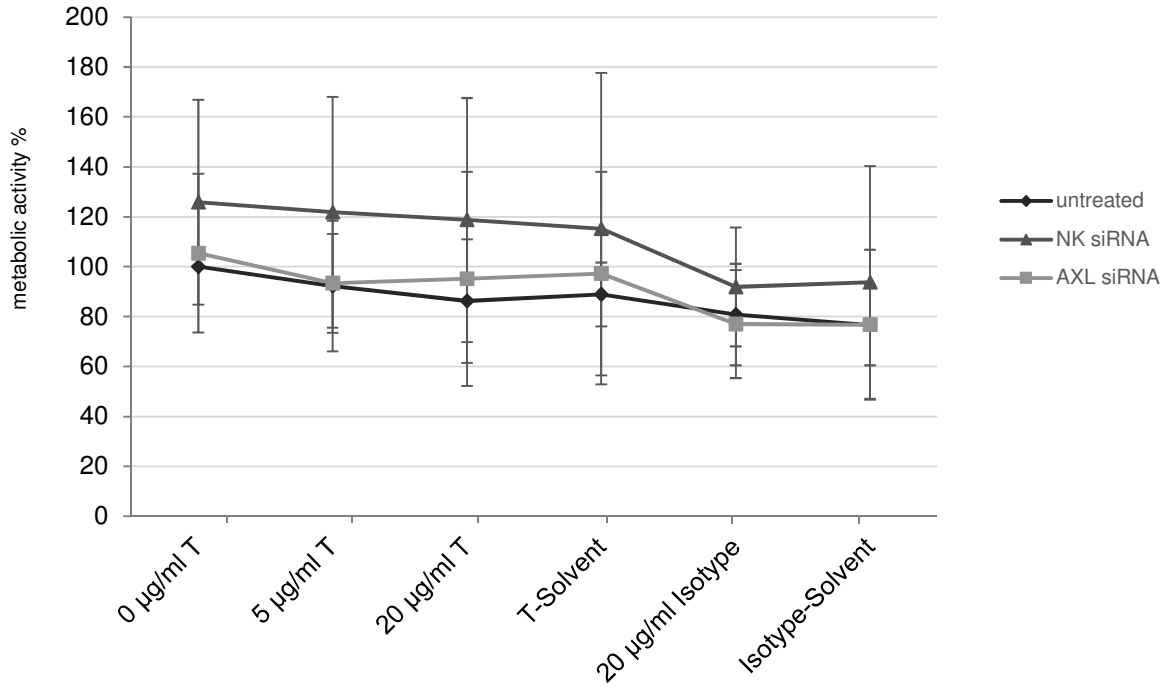


Figure 40: Proliferation analysis of AXL siRNA transfected MKN7.
The effect of the knockdown of AXL was analyzed on the proliferation in the cell line MKN7 under trastuzumab treatment. The proliferation was measured via WST-1 cell proliferation assay in untreated, NK siRNA and AXL siRNA samples. Cells were treated with 5 µg/ml trastuzumab, 20 µg/ml trastuzumab, 20 µg/ml isotype or the appropriate solvent control. All levels were comparable. The untreated sample was set to 100 % to visualize the effect of the different treatments. The mean values of three independent experiments are shown.

3.3.2.2.2 Invasion

The effect of the AXL knockdown on invasiveness was analyzed in MKN7 cells (Figure 41). The effect was investigated in untreated cells and cell treated with 5 µg/ml trastuzumab of untransfected, NK siRNA and AXL siRNA samples. For the untransfected samples no reduction

under trastuzumab occurred. The invasiveness in NK siRNA samples was slightly elevated compared to untransfected cells. Trastuzumab resulted in a small, not-significant reduction of invasion. The cells transfected with 50 pmol AXL siRNA showed a higher ratio of invasive cells than the other samples. The invasion was reduced in the AXL siRNA cells treated with trastuzumab compared to untreated AXL siRNA cells. This effect was not significant.

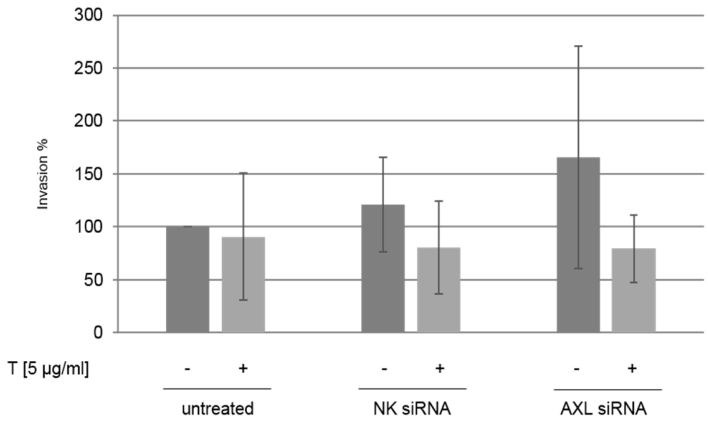


Figure 41: Invasion analysis of AXL siRNA transfected MKN7.
The effect on the invasiveness in the cell line MKN7 was analyzed under trastuzumab and the knockdown of AXL. Under trastuzumab treatment the invasiveness in AXL siRNA samples was slightly reduced. In untreated control samples trastuzumab did not influence the invasiveness. In the negative control (NK siRNA) samples the invasiveness was slightly reduced under trastuzumab treatment. The untreated sample was set to 100 % to visualize the effect of the different treatments. The mean values of three independent experiments are shown.

3.3.2.2.3 Activation and expression of proteins under trastuzumab

The molecular effect of the AXL knockdown was investigated by western blot analysis in the cell line MKN7 (Figure 42). The influence of the knockdown itself by comparing the untreated AXL siRNA cells with control cells was analyzed. Furthermore, untreated, NK siRNA and AXL siRNA cells were treated with trastuzumab and the effect was compared to each other.

For the activation of EGFR and HER2 an increasing effect was detected under trastuzumab treatment in the untransfected, NK siRNA and AXL siRNA samples. The occurring differences in the intensity of the reactions are neglectable. The basal EGFR activation was similar between the control cells and AXL siRNA cells. For HER2 an increased basal activation was detected. No changes occurred in untreated or trastuzumab-treated cells of untransfected, NK siRNA and AXL siRNA samples for pAKT. For the activation of ERK similar levels were detected all showing a reduction of ppERK under trastuzumab treatment. The expression of AXL was not altered by trastuzumab treatment.

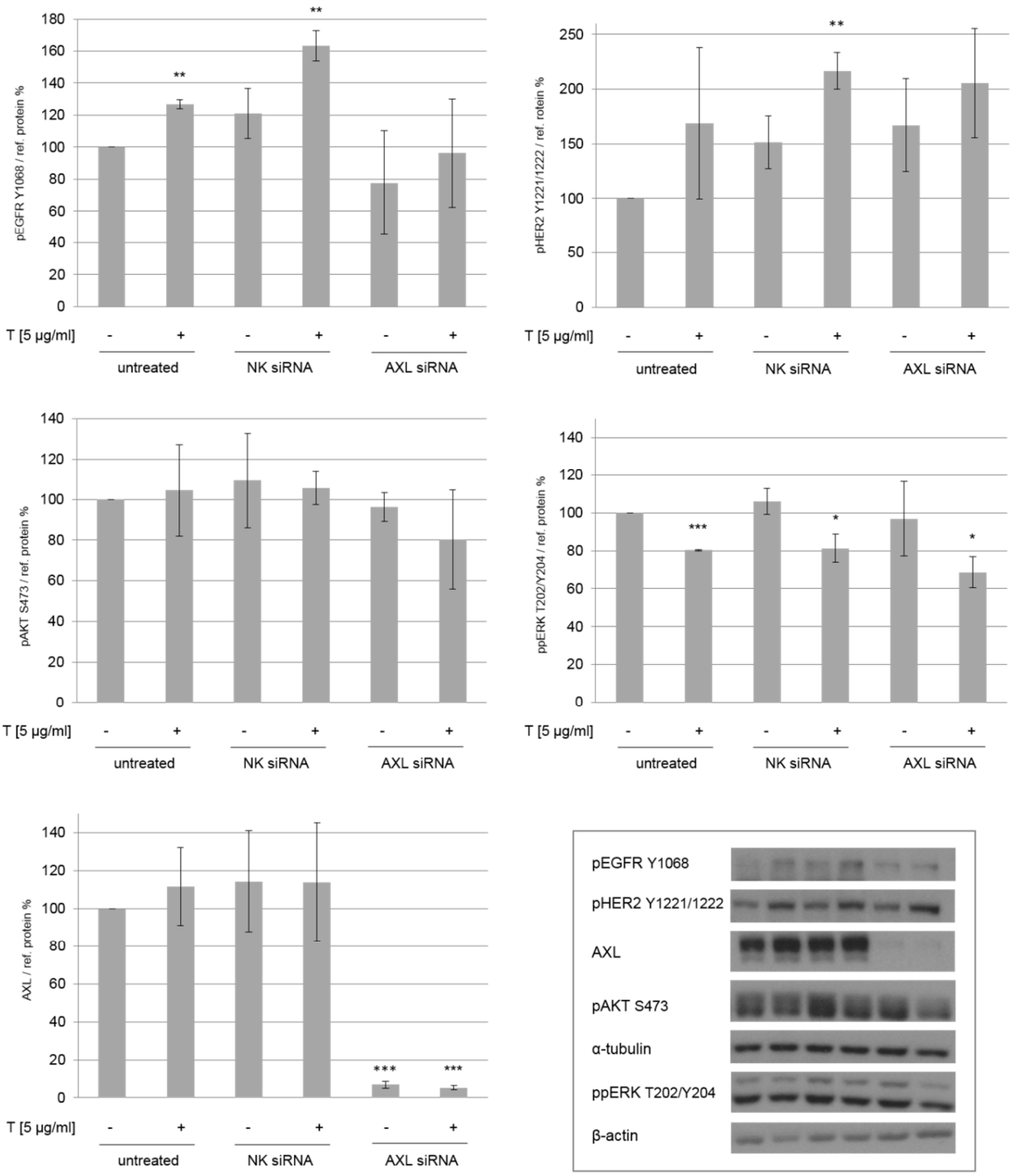


Figure 42: Protein regulation of AXL siRNA-transfected MKN7.
The effect on the activation of EGFR, HER2, AKT, ERK and the expression of AXL was analyzed via Western blotting in MKN7 cells. The regulation under trastuzumab was analyzed in untreated samples, NK siRNA and AXL siRNA samples. The mean values of three independent experiments are shown with one picture as an exemplary result. Significant effects are indicated by * 0.01 < p-value ≤ 0.05, ** 0.001 < p-value ≤ 0.01 or ***< 0.001 (one-sample t-test).

4 DISCUSSION

4.1 Classification of gastric cancer cell lines into trastuzumab responder and non-responder

In the present study, the HER2 signaling pathway in response to trastuzumab treatment was characterized in the 12 gastric cancer cell lines AGS, GSU, H-111-TC, HGC-27, Hs746T, HSC-45, KATO-III, LMSU, MKN1, MKN7, MKN45 and NCI-N87. For trastuzumab, the therapeutic HER2-directed antibody, two different types of mechanistic effects were described. Direct effects can lead to the partial inhibition of downstream signaling, via uncoupling of the ligand-independent HER2-containing dimers, thus reducing the activation of HER2, while indirect anti-tumor effects are activated by ADCC (Clynes et al. 2000, Cuello et al. 2001, Fujimoto-Ouchi et al. 2007, Ghosh et al. 2011, Hata et al. 2013, Junttila et al. 2009, Molina et al. 2001, Sliwkowski et al. 1999, Yakes et al. 2002). In this work, only direct effects of trastuzumab were studied.

Metastases account for approximately 90 % of deaths from solid tumors. During metastatic progression, cells must migrate and invade through the ECM. Thus, migration and invasion are important steps in the formation of metastases (Gupta and Massague 2006, Hanahan and Weinberg 2000). The HER receptors are responsible for cell motion (Friedl and Alexander 2011, Wells et al. 2002), which plays a major role in the invasiveness of tumor cells (Wells 2000, Wells et al. 2013). Migration is subdivided into four phases: adhesion, spreading, contraction and retraction of the cell body (Holly, Larson, and Parise 2000).

The motility behavior was analyzed as phenotypic marker for the trastuzumab sensitivity of all gastric carcinoma cell lines of the panel. For the analysis, different matrix coatings were evaluated and the parameters “motility” and “speed” were detected. It is known that cell adhesion - the first phase of cell migration - is influenced by different matrices. This evaluation is mandatory because every cell line has different adhesion and motility characteristics. These differences are reflected in the different cell morphology and growing patterns. Small and only slightly adherent cell lines such as GSU, KATO-III and MKN45 were hardly motile on any analyzed coating. Cell lines MKN45 and KATO-III harbor mutations in the *CDH1* gene, which impairs motility behavior (Oda et al. 1994). Low motility was detected for H-111-TC, HGC-27 and MKN7 cells. The cell line MKN7 showed delayed attachment of the cells to the substrate. Thus, increased motility of MKN7 cells was achieved by extending the cell culture time from 1 to 24 hours before starting the analysis. The best conditions for all cell lines were achieved by using collagen type I. This coating agent promoted the highest motility in cell lines AGS, Hs746T, HSC-45, LMSU, MKN1 and NCI-N87.

Collagen type I has been previously reported to increase the motility of gastric cancer cells (Li et al. 2010). Collagen type I causes destruction of E-cadherin adhesion complex through the regulation of FAK (Koenig et al. 2006, Menke et al. 2001). Except cadherins, numerous cell-surface proteins such as immunoglobulin superfamily, integrin family, selectins and mucins are involved in cell-adhesion to the ECM, which consists of fibronectin, proteoglycans, heparin, fibrin and collagen type I, III, V and VI (Nollet, Kools, and Roy 2000).

All cell lines were analyzed in an untreated state. Low motility can be elevated by applying EGF, as reported for MKN1 and MKN7 cells (Takeuchi et al. 1996). No significant concentrations of EGF were detected in conditioned cell culture medium or in cell extract (Kneissl et al. 2012).

The HER receptors are also responsible for cell proliferation and survival (Prenzel et al. 2001). To investigate the influence of the HER2 inhibitor trastuzumab on the proliferation of gastric cancer cell lines, a proliferation assay was performed. Cell sensitivity to HER2 inhibitor trastuzumab was tested by determining the viability of gastric cancer cell lines. Only three of the investigated cell lines, GSU, H-111-TC and NCI-N87 showed a significantly reduced metabolic activity under trastuzumab treatment, thus being trastuzumab-sensitive. This effect was not dose-dependent. Trastuzumab-resistant cell lines or cell lines with slight sensitivity to trastuzumab had already been described in other publications (Liu et al. 2015, Tomioka et al. 2012, Wainberg et al. 2010). Although clinically relevant concentrations of trastuzumab were applied, response rates to the treatment were rather low. One reason for the low response rates might be the applied cell culture medium, which contains proliferation-promoting substances. As a result, alternative signaling pathways responsible for cell proliferation or cell survival can be activated, thereby attenuating the inhibitory effect of trastuzumab on HER2 activation.

In order to verify the classification of the gastric cancer cell lines into trastuzumab-sensitive (responder) and trastuzumab-resistant (non-responder) resulting from the proliferation analysis, molecular analyses of the expression and activation of different proteins were carried out. High variations between biological experiments observed in western blot analysis indicate heterogeneity of the cells and sensitivity of the phosphorylation to external factors, as well as slight changes in the cell state. Trastuzumab is a HER2-directed antibody binding HER2 and blocking its activation through phosphorylation. Accordingly, the effect of trastuzumab on HER2 activation of different phosphorylation sites was investigated in all gastric cancer cell lines. In literature, the two most common phosphorylation sites are Y1221/1222 and Y1248 (Ben-Levy et al. 1994, Kawai et al. 2013, Kwon et al. 1997, Segatto et al. 1990, Tomioka et al. 2012). These phosphorylation sites were analyzed in untreated and trastuzumab-treated samples. The expression of pHER2 Y1248 in gastric cancer cell lines had already been analyzed in our research group, by applying

higher concentrations of trastuzumab for a longer time (Kneissl et al. 2017). Despite different treatment conditions, the results previously obtained are comparable to the results presented in this work. The basal activation level of HER2 and the effect of trastuzumab differed across the gastric cancer cell lines. Cell line H-111-TC displayed an increase of the HER2 phosphorylation, whereas KATO-III and NCI-N87 showed a decreased activation under trastuzumab treatment. The effect of trastuzumab in cell line H-111-TC might be caused by compensatory mechanisms or increased stress due to the treatment. Contrary to expectations, this reaction could indicate trastuzumab sensitivity, as it had already been described in trastuzumab-sensitive breast cancer cells, which showed an enhanced activation of HER2 upon trastuzumab therapy (Diermeier et al. 2005, Dokmanovic et al. 2014).

The decrease of HER2 activation under trastuzumab treatment in KATO-III and NCI-N87 identifies the two cell lines as responder cell lines. For the cell line NCI-N87 a response to trastuzumab had already been reported in literature (Kim, Kim, Kim, et al. 2008). All the other cell lines showed inconsistent results on HER2 activation following trastuzumab treatment.

EGFR activation was analyzed in order to identify a possible crosstalk between HER2 and EGFR. Differences in EGFR activation in the gastric cancer cell lines reflect differences of EGFR activation and expression observed in gastric carcinoma patients. No significant changes in EGFR activation were detected in the gastric cancer cell lines under trastuzumab treatment. Based on this result, trastuzumab does not affect EGFR activation or the formation of EGFR-HER2 heterodimers.

Differences in the basal activation of EGFR and HER2 were detected within the gastric cancer cell line panel. Thus, a comprehensive gastric cancer cell line panel was characterized for trastuzumab treatment, reflecting the diversity observed in gastric carcinoma patients. Earlier results of our research group on the basal activation of EGFR and the activation of HER2, detected by analyzing the phosphorylation site Y1248, are consistent with the results obtained in this work (Kneissl et al. 2017).

For all cell lines, HER2 expression was detected at different intensities by western blot analysis. HER2 expression was not detected by IHC analyses in all of the gastric cancer cell lines. The strongest expression of HER2 (score 3+) was found in MKN7 and NCI-N87 cells. These results were confirmed by CISH analysis showing *HER2* amplification in cell lines MKN7 and NCI-N87. Fujimoto-Ouchi et al. reported a score of 2+ for cell line NCI-N87 (Fujimoto-Ouchi et al. 2007). Lower HER2 expression levels were detected for cell lines GSU, H-111-TC (score 2+) and HSC-45 (score 1+). For cell line H-111-TC, but not for GSU, *HER2* amplification was detected by CISH analysis. IHC analyses did not reveal any trastuzumab effect on HER2 expression. In western blot

analysis, cell line KATO-III showed an increased HER2 expression under trastuzumab treatment. This might be due to compensatory mechanisms resulting from trastuzumab-induced pHER2 inhibition. IHC is the standard procedure in the clinic to determine HER2 expression in gastric cancer and to select patients for anti-HER2 treatment with trastuzumab. Only HER2+ gastric cancer patients receive trastuzumab treatment. For this reason, cell lines MKN7 and NCI-N87, which show a strong HER2 expression (score 3+), were chosen for further investigations. Cell line NCI-N87 is a promising responder candidate, as both proliferation and HER2 activation are affected by trastuzumab treatment. Cell line MKN7 did not show any response to trastuzumab in any analysis. In addition, cell line MKN1 was chosen for further investigations because of a reported mutation in *PIKCA*. In breast cancer patients mutations in *PIK3CA* gene have been associated with reduced response to trastuzumab and/or lapatinib neoadjuvant therapy (Majewski et al. 2015). No such mutation was reported for trastuzumab-responder cell line NCI-N87, while it had been previously described that MKN1 harbors a E545K mutation in *PIKCA* (Bamford et al. 2004).

4.2 Analysis of the responder and non-responder cell lines

4.2.1 Phenotypic characterization

In the development of cancer, increased proliferation accompanies tumor growth, and is followed by angiogenesis and epithelial-mesenchymal transition, which enables the cells to leave their place and enter blood and lymph vessels (Alberts, Schäfer, and Häcker 2011). Motility as a measure of cell mobility is another characteristic of malignant cancer cells (Gupta and Massague 2006). *In vitro* analyses of motility in the absence of an attractive concentration gradient had been described as random walk (Wells et al. 2013). In this work, cell motility was determined by surrogate markers *percentage of motile cells* and *speed*. The influence of trastuzumab was analyzed in cell lines MKN1, MKN7 and NCI-N87.

For MKN1 cells collagen type I was used as matrix. Trastuzumab did not have any effect in MKN1 cells. In MKN7 there was only a slight reduction of speed and motility under trastuzumab treatment, however this effect was not significant. These inhibitory effects of trastuzumab may be partially explained by the use of laminin as matrix. In fact, the interaction of tumor cells with laminin leads to intracellular signaling via protein kinases, phosphatases and GTPases. Therefore, laminin can affect the cytoskeleton, and the invasion and metastasis processes (Givant-Horwitz, Davidson, and Reich 2005). The molecules of the extracellular matrix, including laminin, protect breast cancer cells from chemically induced apoptosis (Weaver et al. 2002, Zutter 2007). For the cell line NCI-N87 no significant reduction in motility and speed was detected under trastuzumab treatment. Possible small effects of trastuzumab are masked by a high variability observed between biological experiments. The manual evaluation of time-lapse microscopy films revealed strong fluctuations in motility and speed of individual cells; therefore, results were not statistically significant. Tumor cells themselves are of a very heterogeneous nature, differing greatly in speed and motility. In a study of another research group it was shown that cells display different motility behaviors which are dependent on the cell cycle phases (Hartmann-Petersen et al. 2000). In this study the cell cycle of the examined cells was not synchronized before the motility analysis. Time-lapse microscopy is very sensitive to interference, due to the high complexity of cell motion.

Another way to establish trastuzumab sensitivity in gastric cancer cell lines MKN1, MKN7 and NCI-N87 was by investigating the effect of trastuzumab on cell proliferation. This was achieved by using a WST assay.

Comparing the results of the cell lines MKN1, MKN7 and NCI-N87, a different response to trastuzumab is obvious. The treatment with trastuzumab of cell lines MKN1 and MKN7 did not result in decreased metabolic activity. A slight increasing effect of trastuzumab on metabolic

activity was detected in both cell lines, which might result from treatment-induced stress. Thus, in regard to proliferation, cell lines MKN1 and MKN7 can be classified as non-responder to trastuzumab. Cell line NCI-N87 responds to trastuzumab, by showing a reduced metabolic activity to 90 %, compared to untreated cells. No stronger decrease of the metabolic activity is observed under increasing concentrations of trastuzumab, therefore the inhibitory effect is concentration independent. Published data on the effects of trastuzumab on proliferation in NCI-N87 cells vary greatly (Fujimoto-Ouchi et al. 2007, Garner et al. 2013, Kim, Kim, Kim, et al. 2008, Ko et al. 2015, Patel et al. 2009, Yamashita-Kashima et al. 2011, Yokoyama et al. 2006). Kim et al. and Patel et al. showed a concentration-independent decrease in proliferation under trastuzumab to ~60 % (Kim et al. 2007, Patel et al. 2009). Ko et al. described a concentration-dependent decrease to 80 % (Ko et al. 2015); whereas Yokoyama et al. detected a growth inhibition of NCI-N87 cells to 50 % under trastuzumab treatment (Yokoyama et al. 2006).

HER signaling pathway and its downstream signaling pathway molecules play an important role in the induction of migration and invasion (Wells et al. 2002). In addition to motility and proliferation, the invasion ability of MKN1, MKN7 and NCI-N87 was analyzed to complete the phenotypic analysis. For all cell lines only a small percentage of the applied cells in the invasion assay was actually invasive. High variations between biological experiments occurred, indicating that invasion is sensitive to external factors and slight changes in the cell state. Nevertheless, a significant decrease in the number of invasive cells was observed under trastuzumab treatment in cell line NCI-N87 compared to untreated cells. Thus, concerning invasion, NCI-N87 cell line is a trastuzumab responder. No decreasing effect of trastuzumab on invasive cells was observed in cell lines MKN1 and MKN7. In MKN1 cells a small increase in the number of invasive cells was detected under trastuzumab treatment, compared to untreated cells, which might be due to treatment-induced stress. Both cell lines are non-responder to trastuzumab treatment. This resistance behavior of MKN7 cells to trastuzumab had already been described in the literature (Tomioka et al. 2012).

4.2.2 Molecular characterization

As already known, the receptor density on the cell surface is not statically determined, but regulated by a dynamic process of endo- / exocytosis and degradation. Thus, cells can control and adapt their sensitivity to external stimuli in a condition- and time-dependent manner. Blocked receptors are endocytosed into the cell and lysosomally degraded (Alwan, van Zoelen, and van Leeuwen 2003, Kesarwala, Samrakandi, and Piwnica-Worms 2009).

Klapper et al. demonstrated HER2 internalization and degradation under trastuzumab treatment, by enhancing the activity of the tyrosine kinase ubiquitin ligase c-Cbl (Klapper et al. 2000). However, the molecular mechanisms of trastuzumab-induced c-Cbl recruitment and the mechanisms of HER2 degradation have not yet been fully clarified. No decrease in total HER2 expression was observed in the gastric cancer cell lines under trastuzumab treatment. This result is in line with the results observed in FFPE cell pellets obtained in experiments with longer treatment.

The direct comparison of cell lines MKN1, MKN7 and NCI-N87 revealed different levels of HER2 activation and expression. In NCI-N87 cells, HER2 expression and activation was stronger than in MKN7 cells. For both cell lines a *HER2* amplification had been formerly reported (Fujimoto-Ouchi et al. 2007, Neve, Lane, and Hynes 2001, Tanizaki et al. 2010, Tanner et al. 2005, Tomioka et al. 2012). The strong activation of HER2 in NCI-N87 can be explained by HER2 overexpression resulting in activated HER2-homodimers (Yokoyama et al. 2006). In comparison to NCI-N87, minor levels of activated HER2 were detected in cell line MKN7. Comparing MKN7 to MKN1 cells, a stronger activation and expression of HER2 was observed in MKN7. For MKN1 barely any activation and expression was detected due to low basal HER2 expression. In fact, no *HER2* amplification had been reported for cell line MKN1 (Yokozaki 2000).

No effect of trastuzumab on HER2 activation was observed in cell lines MKN1 and MKN7. For cell line NCI-N87 a small decreasing effect of trastuzumab treatment was observed.

The cell lines were further analyzed independently for HER2 activation, in order to investigate the effect of trastuzumab more precisely. The intensities of HER2 in MKN1 and NCI-N87 cells were comparable to the signals obtained in the direct comparison of the cell lines. Almost no HER2 activation was observed in MKN1. A basal activation of HER2 in cell line MKN1 had been previously reported by using a pan-antibody in a RTK proteome profiler (Gong et al. 2010). For cell line MKN7, a stronger HER2 activation could be detected by increasing the exposure time of the membrane. Nevertheless, no effect of trastuzumab was observed in MKN7. MKN7 cells have been described in literature as resistant to the monoclonal antibody 4D5, which has the identical antigen-binding fragment as trastuzumab (Lane et al. 2000). This resistance could be due also to

alternative signaling by overexpressing EGFR (Neve, Lane, and Hynes 2001, Tomioka et al. 2012). Trastuzumab treatment resulted in a significant decrease of HER2 activation in cell line NCI-N87, as already reported (Janjigian et al. 2013, Kim, Kim, Kim, et al. 2008, Leto et al. 2015). As trastuzumab is able to reduce the activation of HER2, the antibody can block the dimerization of HER2 with itself and with other HER receptors.

The basal activation of HER2 in MKN7 and NCI-N87 is probably due to HER2 overexpression resulting in activated HER2-homodimers and ligand-independent heterodimers (Patel et al. 2009). An activation of HER2 under basal condition in MKN7 cells had already been described using a pan-antibody detecting HER2 activation (Gong et al. 2010).

The receptor EGFR is important for different signaling pathways involved in the regulation of cell proliferation, motility and invasion (Harari, Allen, and Bonner 2007, Zandi et al. 2007). Overexpression or amplification of EGFR in carcinomas is often associated with advanced tumor status, resistance to therapy, and poor prognosis (Arteaga 2002, Holbro, Civenni, and Hynes 2003, Prenzel et al. 2001).

In untreated condition, different EGFR activation levels had been observed for the gastric cancer cell lines, as reported earlier (Guo et al. 2011, Heindl et al. 2012, Kneissl et al. 2017). Overexpression of EGFR was detected in cell lines MKN7 and NCI-N87, which is in line with the results published for MKN7 (Lane et al. 2000, Yokoyama et al. 2006). Tanner and colleagues (Tanner et al. 2005) showed that the expression level of EGFR was much lower than HER2, which fits with the data presented in this work. The detection of basal levels of activated EGFR without ligand treatment might be due to the utilization of complete cell culture medium, containing activating ligands. Influence of trastuzumab on EGFR activation might be observed by the inhibition of HER2 and therefore the restricted formation of EGFR-HER2 heterodimers. In all cell lines no significant effect of trastuzumab was observed. However, in this work trastuzumab did not affect EGFR activation, through the inhibition of ligand-independent EGFR-HER2 heterodimers, in any of the gastric cancer cell lines. A further association of trastuzumab and EGFR described for the cell line MKN7 was not observed in this work. The formation of EGFR-HER3 heterodimers under trastuzumab treatment had been shown to compensate treatment effects, explaining the resistance of MKN7 cells to trastuzumab treatment (Neve, Lane, and Hynes 2001, Tomioka et al. 2012).

The downstream signaling pathways of HER2 and the other HER receptors regulate the processes of tumor progression, such as invasion, migration and proliferation (Johnson et al. 2010, Scaltriti, Elkabets, and Baselga 2016, Vouri et al. 2016). Amongst the pathways that are frequently regulated, Ras ERK and PI3K-AKT signaling pathways have been thoroughly studied (Johnson et

al. 2010, Scaltriti, Elkabets, and Baselga 2016). In general, Ras-ERK regulates proliferation whilst PI3K-AKT signaling pathway regulates cell survival. Of central importance in these signaling pathways are the proteins ERK and AKT, which activate or inhibit further downstream kinases. The activation of these downstream kinases and the effect of trastuzumab were analyzed in the three gastric cancer cell lines. As no effect of trastuzumab on the activation of HER2 and EGFR had been observed in cell lines MKN1 and MKN7, no decreasing effect of trastuzumab on the downstream signaling was expected. For cell line MKN7, this is in line with earlier reports (Tomioka et al. 2012). In general for MKN1 cells the kinases AKT and ERK might be regulated independently of HER receptors, due to the reported *KRAS* amplification and *PIK3CA* (E545K) mutation (Bamford et al. 2004). Cell line NCI-N87 did show a response to trastuzumab at the receptor level, therefore a response in the downstream signaling was also expected. However, trastuzumab did not affect the activation of AKT in the cell line NCI-N87. No molecular effects of trastuzumab were observed in earlier reports investigating the activation of ERK and AKT (Tomioka et al. 2012, Wainberg et al. 2010, Wang et al. 2016, Zheng et al. 2014). The AKT-signaling is promoted by all HER receptors, thus the inhibition of one receptor (HER2) by trastuzumab was insufficient to reduce this signaling in the cell line NCI-N87. Interestingly, trastuzumab is inducing the activity of ERK. This might be due to a rapid reaction of the cell to trastuzumab treatment to compensate the reduced signal from the HER2 receptor. In literature, no effect (Leto et al. 2015, Wainberg et al. 2010, Wang et al. 2016, Zheng et al. 2014) or only a slight reduction (Kim, Kim, Kim, et al. 2008, Yamashita-Kashima et al. 2011) of ERK phosphorylation had been observed under trastuzumab treatment.

Clinically, drugs such as afatinib are administered daily, while others like trastuzumab are administered at intervals of weeks (Bang et al. 2010, Eskens et al. 2008, Soria et al. 2015, Swain et al. 2015). For this reason, kinetic studies with trastuzumab were performed to determine the optimal treatment time for cell culture experiments.

In cell line NCI-N87 the activation of HER2 was significantly decreased comparing the untreated sample with treatment durations of 10 minutes, 4 hours, 7 hours and 22 hours. Comparing the effect observed in the long-treatment samples (7 hours and 22 hours) with their appropriate control sample no significant decreasing trastuzumab effect was observed. Therefore, trastuzumab treatment duration between 10 minutes and 4 hours was the most effective for NCI-N87 cells. In the kinetic analysis, the activation of EGFR was affected by trastuzumab in cell line NCI-N87. A significantly reduced activation was detected in the samples treated for 30 minutes, 1 hour and 4 hours. This reduced activation of EGFR might be due to a reduced formation of EGFR-HER2 heterodimers. This effect was not observed for short treatment durations (5 minutes). This might

be explained by a delayed trastuzumab effect on pHER2 and therefore on the activation of the dimerization partner. The activation of AKT was not significantly altered, when comparing treated samples with the appropriate untreated sample of NCI-N87 cells. An increased activation of ERK under trastuzumab treatment was observed, confirming former results.

In MKN1 cells, trastuzumab did not affect the activation of HER2, EGFR, ERK and AKT. Analysis of different untreated samples revealed a culture time-dependent decreasing effect on the activation of ERK and AKT. This decrease in activation might be due to a reduced availability of growth factors in the cell culture medium. In general, low activation levels were detected for HER2 and EGFR.

The activation of HER2 and AKT was not affected by trastuzumab in MKN7 cells. Small effects of trastuzumab were detected on the activation of EGFR, but this result was not confirmed when treated cells were compared to the corresponding untreated samples. This was also true for ERK activation. An exception was the decrease in ERK activation observed in the sample treated for 30 minutes with trastuzumab.

As significant effects have been observed for cell line NCI-N87 under trastuzumab treatment, the activation of further receptors was investigated. A decreasing effect of trastuzumab on HER2 activation was observed on the phosphorylation site Y1248. This is in line with the data showing the effect of trastuzumab on the HER2 phosphorylation site Y1221/1222. The detection of basal activation of the receptors HER3 and MET was limited, as it had been reported for HER3 (Yokoyama et al. 2006, Tanner et al. 2005). A previously reported MET overexpression (Liu et al. 2011) could not be confirmed by experiments carried out in this work. No effect of trastuzumab was detectable. Basal activation of HER3 and MET might be due to the cell culture media containing activating ligands. The receptor HER3 can be activated by the formation of ligand-independent HER2-HER3 heterodimers due to HER2 overexpression. This possibility can be excluded because trastuzumab was not able to inhibit these heterodimers, thus HER3 activation should have decreased under trastuzumab treatment. These results conflict with previous reports describing a downregulation of HER3 activity in this cell line following trastuzumab treatment (Garner et al. 2013, Leto et al. 2015). Corresponding analyses were not performed on cell line MKN7, as HER3 expression and activation had been previously reported as undetectable (Kneissl et al. 2017, Lane et al. 2000).

4.2.2.1 Proteome Profiler Analyses

HER2-positive gastric carcinoma patients are treated with trastuzumab, with a positive outcome of therapy being achieved in less than 50 % of the cases (Apicella, Corso, and Giordano 2017). Therefore, it can be assumed that the HER2 signaling pathway is bypassed by other RTKs, compensating for the blockade of HER2 by trastuzumab. A combination of two or more agents may be appropriate to achieve a better response in gastric carcinoma. Therefore, afatinib for combination therapy with trastuzumab was evaluated for its effect in gastric cancer cell lines MKN1, MKN7 and NCI-N87. Since the activation of EGFR and HER3 is being discussed as resistance mechanism for HER2-based therapy, EGF was used to detect possible activation of the receptors (Garrett et al. 2011, Rexer and Arteaga 2013, Ritter et al. 2007). It is known that cells can compensate the inhibition of a pathway by up-regulating other signaling pathways, thereby balancing inhibitory effects with redundant pathways (Garrett et al. 2011). Thus, the RTK proteome profiler was used to identify the activation of 49 of the 58 known RTKs of the human genome (Robinson, Wu, and Lin 2000). Furthermore, a kinase proteome profiler was used to identify regulations in different downstream signaling pathways. This work had been published by Keller, Zwingenberger and colleagues (Keller et al. 2018).

For HER2 activation, different trastuzumab response patterns in the different cell lines were found. In cell line NCI-N87, trastuzumab reduced the activation of HER2 as observed in western blot analysis. These results suggest that trastuzumab is able to block HER2-homodimerization and ligand-independent HER2-heterodimerization in this cell line. For NCI-N87 a strong expression and activation of HER2, as well as *HER2* amplification and *HER2* mutations are known (Arienti et al. 2016, Bamford et al. 2004, Tym et al. 2016). In line with western blot results, MKN1 cells did not respond to trastuzumab treatment. A low basal HER2 expression level and HER2 activation observed in this cell line might explain the nonresponse, as reported in literature (Ishida et al. 2016, Wainberg et al. 2010).

In MKN7 cells trastuzumab treatment induced a less pronounced inhibition of HER2 activation than in NCI-N87 cells. MKN7 cells are characterized by a strong expression of HER2 and a slightly lower HER2 activation than NCI-N87 cells, although both cell lines harbor a *HER2* amplification. MKN7 cells reacted similarly to the antibody 4D5, the murine precursor to trastuzumab, which has the same antigen-binding fragment as trastuzumab (Carter et al. 1992, Lane et al. 2000). A possible explanation for the reduced sensitivity of MKN7 cells to trastuzumab includes the shedding of the extracellular trastuzumab-binding domain of HER2 (Molina et al. 2002), which was analyzed in this work.

Despite the different effects of trastuzumab on the activation of HER2, no inhibition of the analyzed downstream signaling kinases was detected in any of the cell lines. As reported in literature, even under higher concentrations of trastuzumab and longer treatment durations, no effects on ERK and inconsistent effects on AKT occurred in cell lines MKN7 and NCI-N87 (Han et al. 2014, Leto et al. 2015, Tanizaki et al. 2010, Wainberg et al. 2010, Zheng et al. 2014).

For cell line MKN1 the insensitivity to trastuzumab might be explained by the low HER2 activation and a strong activation of the receptor ROR2. ROR2 is involved in the regulation of cellular invasiveness and cell motility and is implicated in WNT signal transduction (Green, Nusse, and van Amerongen 2014). High expression of ROR2 was associated with more aggressive disease states in various cancer entities (Debebe and Rathmell 2015). Furthermore, the activating mutation in *PIK3CA*, which is associated with constitutive kinase activation, might be linked to the trastuzumab resistance observed in MKN1 cells (Kang, Bader, and Vogt 2005).

The non-responsiveness to trastuzumab treatment of the downstream kinases in MKN7 and NCI-N87 cells can be potentially explained by the hetero-dimerization of EGFR and HER3 (Neve, Lane, and Hynes 2001) or by persistent HER3 signaling (Motoyama, Hynes, and Lane 2002, Wheeler et al. 2008), which could compensate for HER2 inhibition. ROR2 is also activated in the cell line MKN7. In addition to the potential HER2 shedding in this cell line, another possible cause of resistance is the presence of the activated receptor AXL (Arteaga and Engelman 2014, Huang et al. 2010, Korshunov 2012, Liu et al. 2009, Nahta et al. 2007, Sawabu et al. 2007). Both possible resistance mechanisms were examined in this work. Further biomarker candidates, such as the prostaglandin transporter, were not investigated. High mRNA und protein expression were detected in MKN7 but not in NCI-N87 cells, predicting a poor prognosis associated with angiogenesis in gastric cancer (Takeda et al. 2016).

Indirect immunological effects of trastuzumab might be of high relevance, as anti-tumor effect by triggering ADCC. Accordingly, a significantly reduced tumor volume was observed in a xenograft model with the cell line NCI-N87 under trastuzumab treatment (Matsui et al. 2005).

An alternative treatment option is necessary for gastric cancer patients without *HER2* amplification or overexpression and for patients who develop a resistance to trastuzumab treatment during therapy.

This issue is addressed in clinical trials investigating the effect of afatinib, a pan-HER tyrosine kinase inhibitor (Memorial Sloan Kettering Cancer Center 2017, Wind et al. 2016). In the kinase proteome profiler inhibitory effects of afatinib on the activation of AKT1/2/3, WNK1 and ERK1/2 were detected for the cell line NCI-N87. These effects were even more pronounced in MKN7 cells. Furthermore, inhibitory effects of afatinib were demonstrated for ERK1/2 in the cell line MKN1. All

three cell lines show a different status of HER2 expression and activation. To conclude, afatinib showed inhibitory effects on downstream signaling independent of HER2 expression or activation. NCI-N87 cells were already reported to be sensitive to afatinib by the MTT proliferation assay (Nam et al. 2012) and in xenograft models (Janjigian et al. 2013, Leto et al. 2015). Afatinib is a potential second-line therapy option in HER2-positive gastric cancer and should also be taken into account as a therapeutic option in HER2-negative gastric carcinoma.

Only small effects of trastuzumab in the responder cell line NCI-N87 investigating molecular and phenotypic response were observed. Fujimoto-Ouchi et al. concluded mainly indirect antitumor activity such as ADCC of trastuzumab treatment (Fujimoto-Ouchi et al. 2007). This is in line with the results obtained in NCI-N87 xenograft models showing a reduced tumor volume under trastuzumab treatment (Eskens et al. 2008, Fujimoto-Ouchi et al. 2007, Janjigian et al. 2013, Ko et al. 2015, Leto et al. 2015, Matsui et al. 2005, Patel et al. 2009, Tanner et al. 2005). Furthermore, Arienti and colleagues published the results of genome sequencing of NCI-N87 cells (Arienti et al. 2016). For the cell line NCI-N87 five different HER2 variant and three different HER3 variants are known. The low and variable response of NCI-N87 cells to trastuzumab could be due to the gene variants.

4.3 Validation of resistance factors

4.3.1 HER2 Shedding

HER2 shedding is a resistance mechanism against HER2-targeted therapy in HER2-overexpressing tumor cells. The full-length HER2 receptor undergoes a slow proteolytic cleavage by matrix metalloproteases resulting in different forms of HER2 (Lin and Clinton 1991, Zabrecky et al. 1991). Molina et al showed that trastuzumab is able to block the proteolytic cleavage of HER2 *in vitro* (Molina et al. 2001). The shedded HER2 ECD was detectable in serum (Leitzel et al. 1995, Yamauchi et al. 1997) and in cell culture medium (Lin and Clinton 1991, Pupa et al. 1993). In breast cancer, high levels of HER2 ECD correlate with a poor prognosis (Colomer et al. 2000). The remaining HER2 ICD shows a basal kinase activity (Anido et al. 2006, Christianson et al. 1998). Accordingly, even in the presence of trastuzumab, intracellular HER2 signaling would remain active, confirming the thesis of HER2-ECD-mediated resistance to HER2-directed antibody therapies (Price-Schiavi et al. 2002). For HER2 ICD-expressing breast cancer, a trastuzumab resistance was described (Scaltriti et al. 2007). These tumors require additional or an alternative therapy targeting HER2. The HER2 shedding was analyzed for the three gastric cancer cell lines MKN1, MKN7 and NCI-N87. The HER2 ECD was detected via ELISA, whilst all the forms of HER2 were measured by western blot analysis using a c-terminal-directed antibody. For MKN1 cells, only the full-length receptor was detected. No values were detectable for HER2 ECD, measured by ELISA. Thus, no HER2 shedding occurs in the cell line MKN1. In general, low HER2 expression was measured for the cell line MKN1, with a small decreasing effect of trastuzumab on HER2 expression. The cell line MKN7 mainly showed the full-length HER2 receptor. Small fractions of the shedded forms and HER2 ECD were detected. Over culture time, the overall HER2 expression in MKN7 slightly increased. Accordingly, the detection of extracellular HER2, and therefore HER2 shedding, increased during culture time, an effect that might be due to accumulation. Trastuzumab treatment did not influence the HER2 shedding. The highest levels of HER2 ECD, thus HER2-shedding, were detected in the cell line NCI-N87. In this cell line, all known forms of HER2 were detected. A small decreasing effect of trastuzumab on HER2 shedding was observed. As for MKN7 cells, the detection of extracellular HER2 and therefore HER2 shedding is slightly increased over culture time.

HER2 shedding occurs in the cell line MKN7 and NCI-N87. The culture time increased the shedding of HER2, especially in MKN7. The effect of trastuzumab is minor in both cell lines. Therefore, HER2 shedding is not a biomarker for trastuzumab resistance in MKN7.

4.3.2 AXL

The analysis of the gastric cancer cell lines revealed a strong activation of AXL in the RTK proteome profiler for the cell line MKN7. Since the activation of HER2 is only marginally reduced under trastuzumab and no downstream effects of phosphorylated kinases were detected, it was assumed that AXL could act as a resistance factor to trastuzumab therapy in HER2-positive gastric cancer. AXL could compensate the inhibition of HER2 by trastuzumab via a bypass track thus maintaining the signaling (Arteaga and Engelman 2014, Niederst and Engelman 2013). In breast cancer patients an increased expression of AXL was associated with a negative prognosis (Gjerdrum et al. 2010).

To characterize AXL as a potential biomarker for trastuzumab therapy in gastric cancer, the association of AXL knockdown (kd) with the response to trastuzumab was investigated in the cell line MKN7. The standard method of transient lipo-transfection was chosen. This can be applied to a large number of cell lines, has a high transfection efficiency and, unlike stable transfection, the cells are less affected (Dalby et al. 2004, Kurreck 2009). The results of the establishment experiments with 10, 50 and 75 pmol AXL siRNA showed that the transfection of MKN7 cells significantly reduced the expression of AXL for all analyzed concentrations. The knockdown was stable for 120 hours in all analyzed samples. No non-specific effects were observed by analyzing appropriate controls.

As an optimal siRNA concentration 50 pmol AXL siRNA was chosen. A concentration of 75 pmol AXL siRNA was excluded because high siRNA concentrations can cause non-specific or toxic effects. For the lowest analyzed concentration of 10 pmol AXL siRNA the microscopic determination of transfection efficiency by fluorescence was extremely low.

One of the "hallmarks of cancer" is the maintenance of persistent proliferation (Hanahan and Weinberg 2011) and must be inhibited in order to stop the progression of the disease. A connection between AXL overexpression and increase cell proliferation was reported for prostate carcinoma cell lines (Paccez et al. 2013). Furthermore, in mesothelioma, a connective tissue tumor of the mesothelium, proliferation was inhibited by a knockdown of AXL (Ou et al. 2011). Based on these results, it has been suggested that a knockdown of AXL in MKN7 reduces the proliferation and increases the sensitivity to trastuzumab in this cell line. The obtained results did not confirm this assumption. No significant influence of AXL knockdown on proliferation was detected in transfected cells compared to untransfected cells. Furthermore, trastuzumab did not inhibit the proliferation in the AXL knockdown cells compared to untransfected trastuzumab-treated cells. Small non-significant effects occurred in the control samples. Kneissl et al. showed that

proliferation was unaffected in trastuzumab-treated samples, but small significant decreases were observed in isotope-treated MKN7 cells (Kneissl et al. 2017).

In summary, the proliferation experiment showed that AXL expression does not correlate with the proliferation of the MKN7 cell line or with its response to trastuzumab therapy.

Another hallmark of cancer is the activated invasion as a process that interacts with migration (Hanahan and Weinberg 2011). Therefore, the effect of AXL knockdown on cell invasion was investigated in MKN7 cells, with and without treatment with trastuzumab. The results showed a small decrease in invasion in the trastuzumab-treated AXL kd cells compared to untreated cells. This decrease is more pronounced than the one observed in trastuzumab-treated untransfected cells when compared to untransfected untreated cells. As described, the knockdown of AXL inhibited the invasion of colorectal cancer and breast cancer cell lines (Uribe et al. 2017, Zhang et al. 2008). This result could only be partially confirmed for the gastric carcinoma cell line MKN7, due to non-significant effects.

For the study of the effects of AXL knockdown and trastuzumab therapy on protein regulation, only a selection of receptors and kinases that might be affected was analyzed. Since no effect of trastuzumab on the expression of HER2 and EGFR was observed during the characterization of the cell line, only the activation of the receptors HER2 and EGFR, and the downstream kinases AKT and ERK was analyzed. Increasing effects of trastuzumab on the activation of HER2 were observed in all samples. The knockdown of AXL did not influence the activation of HER2 under trastuzumab treatment compared to untransfected cells. Similar effects were observed for EGFR. Since EGFR is not a target of trastuzumab, a reduced activation under the treatment with trastuzumab was not necessarily expected. Nevertheless, an interaction of EGFR with AXL has been described in literature (Meyer et al. 2013, Vouri et al. 2016). It has been reported that the activation of EGFR can transactivate AXL (Meyer et al. 2013). Here, only AXL expression was detected and no statement can be made about the activation of AXL. Therefore, no direct correlation between AXL expression, HER2 or EGFR activation and trastuzumab response can be determined in the gastric cancer cell line MKN7.

Signaling pathways downstream of the receptors AXL, HER2 and EGFR that are frequently regulated include the Ras ERK pathway, which regulates proliferation, and the PI3K-AKT signaling pathway, which regulates cell survival (Johnson et al. 2010, Scaltriti, Elkabets, and Baselga 2016, Vouri et al. 2016). Of central importance in these signaling pathways is the activation of the proteins ERK and AKT, which activate or inhibit further downstream kinases. For the cell line MKN7, a comparison of untreated and trastuzumab-treated cells did not result in altered activation of AKT and ERK (Tanizaki et al. 2010). This was confirmed by the results obtained for AKT. No

regulation of pAKT was detected in all analyzed samples. These results conflict with the described inhibition of pAKT, when AXL was inhibited (Sawabu et al. 2007). Other RTKs could possibly compensate the inhibition of pAKT via AXL.

AXL knockdown did not affect pERK; consequently, no correlation between AXL and the activation of the kinases AKT and ERK was observed.

The initial hypothesis that AXL is a resistance factor for trastuzumab in MKN7 cells has proven false for both criteria of proliferation and regulation of the Ras-ERK and PI3K-AKT signaling pathways. Although small decreases were observed in the invasion analyses, these changes were not statistically significant. The trastuzumab resistance described for other tumors in connection with the expression of AXL cannot be confirmed for the gastric cancer cell line MKN7.

In the complex network of signaling pathways, the inhibition of trastuzumab might not only be compensated by AXL but multiple receptors (Agarwal et al. 2009, Stommel et al. 2007).

5 CONCLUSION

Therapeutic response to trastuzumab was evaluated in a comprehensive gastric cancer cell line panel by phenotypic characterization and molecular analysis. Response to trastuzumab differed greatly across cell lines. Cell lines MKN1 and MKN7 were identified as non-responder, whereas NCI-N87 was identified as responder to trastuzumab. Molecular and phenotypical effects of trastuzumab therapy were analyzed in detail in the three cell lines. In the responder cell line NCI-N87 small but significant effects under trastuzumab therapy were observed. It remains unclear why the effect of trastuzumab on HER2 was not transduced to the downstream tyrosine kinases, as observed in proteome profiling data. The HER2 overexpression observed in NCI-N87 and MKN7 cells is not critical for the response to trastuzumab, but rather the HER2 activation status. HER2 was activated only in NCI-N87 cells.

In this work, two potential resistance factors to trastuzumab therapy were identified. None of these two factors could be confirmed. No correlation between HER2 shedding and trastuzumab treatment was observed in any of the cell lines analyzed. Nevertheless, HER2 shedding is known to correlate with a poor prognosis and trastuzumab resistance in breast cancer and should be considered as potential mechanism of resistance in gastric cancer patients too. The hypothesis of a correlation between AXL expression and trastuzumab response in the cell line MKN7 could not be confirmed either. AXL knockdown alone or in association with trastuzumab showed no effect in phenotypic and molecular analyses.

Further proteomic and phenotypic analyses are required to confirm any potential biomarkers to trastuzumab therapy identified so far.

The inhibiting effect of afatinib treatment in gastric cancer cell lines was shown in a broad proteome analysis. The strong inhibiting effects of afatinib were independent of HER2 in all gastric cancer cell lines. Compared to afatinib monotherapy, the combination of afatinib with trastuzumab did not result in enhanced response of the downstream kinases. Further investigations are recommended in order to establish whether afatinib could be a feasible treatment option for trastuzumab-resistant gastric cancer patients.

6 LITERATURE

Abercrombie, M., G. A. Dunn, and J. P. Heath. 1977. "The shape and movement of fibroblasts in culture." *Soc Gen Physiol Ser* 32:57-70.

Adams, G. P., and L. M. Weiner. 2005. "Monoclonal antibody therapy of cancer." *Nat Biotechnol* 23 (9):1147-57. doi: 10.1038/nbt1137.

Adams, J. C. 2001. "Cell-matrix contact structures." *Cell Mol Life Sci* 58 (3):371-92. doi: 10.1007/PL00000864.

Agarwal, S., C. Zerillo, J. Kolmakova, J. G. Christensen, L. N. Harris, D. L. Rimm, M. P. Digiovanna, and D. F. Stern. 2009. "Association of constitutively activated hepatocyte growth factor receptor (Met) with resistance to a dual EGFR/Her2 inhibitor in non-small-cell lung cancer cells." *Br J Cancer* 100 (6):941-9. doi: 10.1038/sj.bjc.6604937.

Agus, D. B., R. W. Akita, W. D. Fox, G. D. Lewis, B. Higgins, P. I. Pisacane, J. A. Lofgren, C. Tindell, D. P. Evans, K. Maiese, H. I. Scher, and M. X. Sliwkowski. 2002. "Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth." *Cancer Cell* 2 (2):127-37.

Akagi, T., and T. Kimoto. 1976. "Human cell line (HGC-27) derived from the metastatic lymph node of gastric cancer." *Acta Med Okayama* 30 (3):215-9.

Alberts, Bruce, Ulrich Schäfer, and Bärbel Häcker. 2011. *Molekularbiologie der Zelle.* Edited by 5. Aufl. Weinheim: Wiley-VCH.

Alwan, H. A., E. J. van Zoelen, and J. E. van Leeuwen. 2003. "Ligand-induced lysosomal epidermal growth factor receptor (EGFR) degradation is preceded by proteasome-dependent EGFR de-ubiquitination." *J Biol Chem* 278 (37):35781-90. doi: 10.1074/jbc.M301326200.

Anido, J., M. Scaltriti, J. J. Bech Serra, B. Santiago Josefat, F. R. Todo, J. Baselga, and J. Arribas. 2006. "Biosynthesis of tumorigenic HER2 C-terminal fragments by alternative initiation of translation." *EMBO J* 25 (13):3234-44. doi: 10.1038/sj.emboj.7601191.

Apicella, M., S. Corso, and S. Giordano. 2017. "Targeted therapies for gastric cancer: failures and hopes from clinical trials." *Oncotarget* 8 (34):57654-57669. doi: 10.18632/oncotarget.14825.

Arienti, C., M. Zanoni, S. Pignatta, A. Del Rio, S. Carloni, M. Tebaldi, G. Tedaldi, and A. Tesei. 2016. "Preclinical evidence of multiple mechanisms underlying trastuzumab resistance in gastric cancer." *Oncotarget* 7 (14):18424-39. doi: 10.18632/oncotarget.7575.

Armstrong, B., and R. Doll. 1975. "Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practices." *Int J Cancer* 15 (4):617-31.

Arteaga, C. L. 2002. "Epidermal growth factor receptor dependence in human tumors: more than just expression?" *Oncologist* 7 Suppl 4:31-9.

Arteaga, C. L., and J. A. Engelman. 2014. "ERBB receptors: from oncogene discovery to basic science to mechanism-based cancer therapeutics." *Cancer Cell* 25 (3):282-303. doi: 10.1016/j.ccr.2014.02.025.

Bakir, T., G. Can, S. Erkul, and C. Siviloglu. 2000. "Stomach cancer history in the siblings of patients with gastric carcinoma." *Eur J Cancer Prev* 9 (6):401-8.

Bamford, S., E. Dawson, S. Forbes, J. Clements, R. Pettett, A. Dogan, A. Flanagan, J. Teague, P. A. Futreal, M. R. Stratton, and R. Wooster. 2004. "The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website." *Br J Cancer* 91 (2):355-8. doi: 10.1038/sj.bjc.6601894.

Bang, Y. J., E. Van Cutsem, A. Feyereislova, H. C. Chung, L. Shen, A. Sawaki, F. Lordick, A. Ohtsu, Y. Omuro, T. Satoh, G. Aprile, E. Kulikov, J. Hill, M. Lehle, J. Ruschoff, Y. K. Kang, and G. A. Trial Investigators To. 2010. "Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial." *Lancet* 376 (9742):687-97. doi: 10.1016/S0140-6736(10)61121-X.

Baselga, J. 2002. "A new anti-ErbB2 strategy in the treatment of cancer: prevention of ligand-dependent ErbB2 receptor heterodimerization." *Cancer Cell* 2 (2):93-5.

Baselga, J., and S. M. Swain. 2009. "Novel anticancer targets: revisiting ERBB2 and discovering ERBB3." *Nat Rev Cancer* 9 (7):463-75. doi: 10.1038/nrc2656.

Ben-Levy, R., H. F. Paterson, C. J. Marshall, and Y. Yarden. 1994. "A single autophosphorylation site confers oncogenicity to the Neu/ErbB-2 receptor and enables coupling to the MAP kinase pathway." *EMBO J* 13 (14):3302-11.

Berns, K., H. M. Horlings, B. T. Hennessy, M. Madiredjo, E. M. Hijmans, K. Beelen, S. C. Linn, A. M. Gonzalez-Angulo, K. Stemke-Hale, M. Hauptmann, R. L. Beijersbergen, G. B. Mills, M. J. van de Vijver, and R. Bernards. 2007. "A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer." *Cancer Cell* 12 (4):395-402. doi: 10.1016/j.ccr.2007.08.030.

Boehringer, Ingelheim. 2017. "Phase I Trial of Afatinib and Trastuzumab in HER2 Overexpressing Cancer.".

Boku, N. 2014. "HER2-positive gastric cancer." *Gastric Cancer* 17 (1):1-12. doi: 10.1007/s10120-013-0252-z.

Bornschein, J., and P. Malfertheiner. 2011. "Gastric carcinogenesis." *Langenbecks Arch Surg* 396 (6):729-42. doi: 10.1007/s00423-011-0810-y.

Borrmann, R. 1926. *Geschwülste des Magens und Duodenums*. Heidelberg: Springer Berlin.

Boyd, J. A., and J. C. Barrett. 1990. "Genetic and cellular basis of multistep carcinogenesis." *Pharmacol Ther* 46 (3):469-86.

Bray, F., J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre, and A. Jemal. 2018. "Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries." *CA Cancer J Clin*. doi: 10.3322/caac.21492.

Brennan, P. J., T. Kumagai, A. Berezov, R. Murali, and M. I. Greene. 2000. "HER2/neu: mechanisms of dimerization/oligomerization." *Oncogene* 19 (53):6093-101. doi: 10.1038/sj.onc.1203967.

Brown, M., J. R. Black, R. Sharma, J. Stebbing, and D. J. Pinato. 2016. "Gene of the month: Axl." *J Clin Pathol* 69 (5):391-7. doi: 10.1136/jclinpath-2016-203629.

Burridge, K., and M. Chrzanowska-Wodnicka. 1996. "Focal adhesions, contractility, and signaling." *Annu Rev Cell Dev Biol* 12:463-518. doi: 10.1146/annurev.cellbio.12.1.463.

Cameron, D., M. Casey, M. Press, D. Lindquist, T. Pienkowski, C. G. Romieu, S. Chan, A. Jagiello-Gruszfeld, B. Kaufman, J. Crown, A. Chan, M. Campone, P. Viens, N. Davidson, V. Gorbounova, J. I. Raats, D. Skarlos, B. Newstat, D. Roychowdhury, P. Paoletti, C. Oliva, S. Rubin, S. Stein, and C. E. Geyer. 2008. "A phase III randomized comparison of lapatinib plus capecitabine versus capecitabine alone in women with advanced breast cancer that has progressed on trastuzumab: updated efficacy and biomarker analyses." *Breast Cancer Res Treat* 112 (3):533-43. doi: 10.1007/s10549-007-9885-0.

Cancer Genome Atlas Research, Network. 2014. "Comprehensive molecular characterization of gastric adenocarcinoma." *Nature* 513 (7517):202-9. doi: 10.1038/nature13480.

Capdevila, J., E. Elez, T. Macarulla, F. J. Ramos, M. Ruiz-Echarri, and J. Tabernero. 2009. "Anti-epidermal growth factor receptor monoclonal antibodies in cancer treatment." *Cancer Treat Rev* 35 (4):354-63. doi: 10.1016/j.ctrv.2009.02.001.

Cappuzzo, F., M. Varella-Garcia, H. Shigematsu, I. Domenichini, S. Bartolini, G. L. Ceresoli, E. Rossi, V. Ludovini, V. Gregorc, L. Toschi, W. A. Franklin, L. Crino, A. F. Gazdar, P. A. Bunn, Jr., and F. R. Hirsch. 2005. "Increased HER2 gene copy number is associated with response to gefitinib therapy in epidermal growth factor receptor-positive non-small-cell lung cancer patients." *J Clin Oncol* 23 (22):5007-18. doi: 10.1200/JCO.2005.09.111.

Carpenter, G. 1987. "Receptors for epidermal growth factor and other polypeptide mitogens." *Annu Rev Biochem* 56:881-914. doi: 10.1146/annurev.bi.56.070187.004313.

Carter, P., L. Presta, C. M. Gorman, J. B. Ridgway, D. Henner, W. L. Wong, A. M. Rowland, C. Kotts, M. E. Carver, and H. M. Shepard. 1992. "Humanization of an anti-p185HER2 antibody for human cancer therapy." *Proc Natl Acad Sci U S A* 89 (10):4285-9.

Cho, H. S., K. Mason, K. X. Ramyar, A. M. Stanley, S. B. Gabelli, D. W. Denney, Jr., and D. J. Leahy. 2003. "Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab." *Nature* 421 (6924):756-60. doi: 10.1038/nature01392.

Choi, Y. Y., J. M. Bae, J. Y. An, I. G. Kwon, I. Cho, H. B. Shin, T. Eiji, M. Aburahmah, H. I. Kim, J. H. Cheong, W. J. Hyung, and S. H. Noh. 2014. "Is microsatellite instability a prognostic marker in gastric cancer? A systematic review with meta-analysis." *J Surg Oncol* 110 (2):129-35. doi: 10.1002/jso.23618.

Chon, S. H., F. Berlth, P. S. Plum, T. Herbold, H. Alakus, R. Kleinert, S. P. Moenig, C. J. Bruns, A. H. Hoelscher, and H. J. Meyer. 2017. "Gastric cancer treatment in the world: Germany." *Transl Gastroenterol Hepatol* 2:53. doi: 10.21037/tgh.2017.05.07.

Christianson, T. A., J. K. Doherty, Y. J. Lin, E. E. Ramsey, R. Holmes, E. J. Keenan, and G. M. Clinton. 1998. "NH2-terminally truncated HER-2/neu protein: relationship with shedding of the extracellular domain and with prognostic factors in breast cancer." *Cancer Res* 58 (22):5123-9.

Ciardiello, F., and G. Tortora. 2008. "EGFR antagonists in cancer treatment." *N Engl J Med* 358 (11):1160-74. doi: 10.1056/NEJMra0707704.

Citri, A., K. B. Skaria, and Y. Yarden. 2003. "The deaf and the dumb: the biology of ErbB-2 and ErbB-3." *Exp Cell Res* 284 (1):54-65.

ClinicalTrials, U.S National Institutes of. 2018. Clinical Trails. https://ClinicalTrials.gov.

Clynes, R. A., T. L. Towers, L. G. Presta, and J. V. Ravetch. 2000. "Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets." *Nat Med* 6 (4):443-6. doi: 10.1038/74704.

Codony-Servat, J, J Albanell, JC Lopez-Talavera, J Arribas, and J Baselga. 1999. "Cleavage of the HER2 ectodomain is a pervanadate-activable process that is inhibited by the tissue inhibitor of metalloproteases TIMP-1 in breast cancer cells." *Cancer Res* 59:1196-1201.

Cohen, S., G. Carpenter, and L. King, Jr. 1980. "Epidermal growth factor-receptor-protein kinase interactions. Co-purification of receptor and epidermal growth factor-enhanced phosphorylation activity." *J Biol Chem* 255 (10):4834-42.

Colomer, R., S. Montero, A. Lluch, B. Ojeda, A. Barnadas, A. Casado, B. Massuti, H. Cortes-Funes, and B. Lloveras. 2000. "Circulating HER2 extracellular domain and resistance to chemotherapy in advanced breast cancer." *Clin Cancer Res* 6 (6):2356-62.

Correa, P., and V. W. Chen. 1994. "Gastric cancer." *Cancer Surv* 19-20:55-76.

Correa, P., W. Haenszel, C. Cuello, S. Tannenbaum, and M. Archer. 1975. "A model for gastric cancer epidemiology." *Lancet* 2 (7924):58-60.

Cramer, L. P. 1999. "Organization and polarity of actin filament networks in cells: implications for the mechanism of myosin-based cell motility." *Biochem Soc Symp* 65:173-205.

Cuello, M., S. A. Ettenberg, A. S. Clark, M. M. Keane, R. H. Posner, M. M. Nau, P. A. Dennis, and S. Lipkowitz. 2001. "Down-regulation of the erbB-2 receptor by trastuzumab (herceptin) enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in breast and ovarian cancer cell lines that overexpress erbB-2." *Cancer Res* 61 (12):4892-900.

Cukierman, E., R. Pankov, D. R. Stevens, and K. M. Yamada. 2001. "Taking cell-matrix adhesions to the third dimension." *Science* 294 (5547):1708-12. doi: 10.1126/science.1064829.

Dalby, B., S. Cates, A. Harris, E. C. Ohki, M. L. Tilkins, P. J. Price, and V. C. Ciccarone. 2004. "Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications." *Methods* 33 (2):95-103. doi: 10.1016/j.ymeth.2003.11.023.

Datasheet. AGS. "https://www.sigmaaldrich.com/catalog/product/sigma/cb_89090402?lang=de&region=DE."

Datasheet. LMSU. "http://www2.brc.riken.jp/lab/cell/detail.cgi?cell_no=RCB1062."

David, L., R. Seruca, J. M. Nesland, P. Soares, F. Sansonetty, R. Holm, A. L. Borresen, and M. Sobrinho-Simoes. 1992. "c-erbB-2 expression in primary gastric carcinomas and their metastases." *Mod Pathol* 5 (4):384-90.

De Luca, A., M. R. Maiello, A. D'Alessio, M. Pergameno, and N. Normanno. 2012. "The RAS/RAF/MEK/ERK and the PI3K/AKT signalling pathways: role in cancer pathogenesis and implications for therapeutic approaches." *Expert Opin Ther Targets* 16 Suppl 2:S17-27. doi: 10.1517/14728222.2011.639361.

Debebe, Z., and W. K. Rathmell. 2015. "Ror2 as a therapeutic target in cancer." *Pharmacol Ther* 150:143-8. doi: 10.1016/j.pharmthera.2015.01.010.

Deng, M., D. D. Jing, and X. J. Meng. 2013. "Effect of MUC1 siRNA on drug resistance of gastric cancer cells to trastuzumab." *Asian Pac J Cancer Prev* 14 (1):127-31.

Deng, N., L. K. Goh, H. Wang, K. Das, J. Tao, I. B. Tan, S. Zhang, M. Lee, J. Wu, K. H. Lim, Z. Lei, G. Goh, Q. Y. Lim, A. L. Tan, D. Y. Sin Poh, S. Riahi, S. Bell, M. M. Shi, R. Linnartz, F. Zhu, K. G. Yeoh, H. C. Toh, W. P. Yong, H. C. Cheong, S. Y. Rha, A. Boussioutas, H. Grabsch, S. Rozen, and P. Tan. 2012. "A comprehensive survey of genomic alterations in gastric cancer reveals systematic patterns of molecular exclusivity and co-occurrence among distinct therapeutic targets." *Gut* 61 (5):673-84. doi: 10.1136/gutjnl-2011-301839.

Dey, N., C. Williams, B. Leyland-Jones, and P. De. 2015. "A critical role for HER3 in HER2-amplified and non-amplified breast cancers: function of a kinase-dead RTK." *Am J Transl Res* 7 (4):733-50.

Diermeier, S., G. Horvath, R. Knuechel-Clarke, F. Hofstaedter, J. Szollosi, and G. Brockhoff. 2005. "Epidermal growth factor receptor coexpression modulates susceptibility to Herceptin in HER2/neu overexpressing breast cancer cells via specific erbB-receptor interaction and activation." *Exp Cell Res* 304 (2):604-19. doi: 10.1016/j.yexcr.2004.12.008.

Dillon, R. L., D. E. White, and W. J. Muller. 2007. "The phosphatidyl inositol 3-kinase signaling network: implications for human breast cancer." *Oncogene* 26 (9):1338-45. doi: 10.1038/sj.onc.1210202.

Dittrich, A., H. Gautrey, D. Browell, and A. Tyson-Capper. 2014. "The HER2 Signaling Network in Breast Cancer--Like a Spider in its Web." *J Mammary Gland Biol Neoplasia* 19 (3-4):253-70. doi: 10.1007/s10911-014-9329-5.

Dokmanovic, M., Y. Wu, Y. Shen, J. Chen, D. S. Hirsch, and W. J. Wu. 2014. "Trastuzumab-induced recruitment of Csk-homologous kinase (CHK) to ErbB2 receptor is associated with ErbB2-Y1248 phosphorylation and ErbB2 degradation to mediate cell growth inhibition." *Cancer Biol Ther* 15 (8):1029-41. doi: 10.4161/cbt.29171.

Downward, J., Y. Yarden, E. Mayes, G. Scrace, N. Totty, P. Stockwell, A. Ullrich, J. Schlessinger, and M. D. Waterfield. 1984. "Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences." *Nature* 307 (5951):521-7.

Duraes, C., G. M. Almeida, R. Seruca, C. Oliveira, and F. Carneiro. 2014. "Biomarkers for gastric cancer: prognostic, predictive or targets of therapy?" *Virchows Arch* 464 (3):367-78. doi: 10.1007/s00428-013-1533-y.

Eskens, F. A., C. H. Mom, A. S. Planting, J. A. Gietema, A. Amelsberg, H. Huisman, L. van Doorn, H. Burger, P. Stopfer, J. Verweij, and E. G. de Vries. 2008. "A phase I dose escalation study of BIBW 2992, an irreversible dual inhibitor of epidermal growth factor receptor 1 (EGFR) and 2 (HER2) tyrosine kinase in a 2-week on, 2-week off schedule in patients with advanced solid tumours." *Br J Cancer* 98 (1):80-5. doi: 10.1038/sj.bjc.6604108.

Esteva, F. J., V. Valero, D. Booser, L. T. Guerra, J. L. Murray, L. Pusztai, M. Cristofanilli, B. Arun, B. Esmaeli, H. A. Fritsche, N. Sneige, T. L. Smith, and G. N. Hortobagyi.

2002. "Phase II study of weekly docetaxel and trastuzumab for patients with HER-2-overexpressing metastatic breast cancer." *J Clin Oncol* 20 (7):1800-8.
Eto, K., M. Iwatsuki, M. Watanabe, T. Ishimoto, S. Ida, Y. Imamura, S. Iwagami, Y. Baba, Y. Sakamoto, Y. Miyamoto, N. Yoshida, and H. Baba. 2015. "The sensitivity of gastric cancer to trastuzumab is regulated by the miR-223/FBXW7 pathway." *Int J Cancer* 136 (7):1537-45. doi: 10.1002/ijc.29168.
Fan, Z., Y. Lu, X. Wu, and J. Mendelsohn. 1994. "Antibody-induced epidermal growth factor receptor dimerization mediates inhibition of autocrine proliferation of A431 squamous carcinoma cells." *J Biol Chem* 269 (44):27595-602.
Ferlay, J., I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D. M. Parkin, D. Forman, and F. Bray. 2015. "Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012." *Int J Cancer* 136 (5):E359-86. doi: 10.1002/ijc.29210.
Franklin, M. C., K. D. Carey, F. F. Vajdos, D. J. Leahy, A. M. de Vos, and M. X. Sliwkowski. 2004. "Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex." *Cancer Cell* 5 (4):317-28.
Frederick, L., X. Y. Wang, G. Eley, and C. D. James. 2000. "Diversity and frequency of epidermal growth factor receptor mutations in human glioblastomas." *Cancer Res* 60 (5):1383-7.
Friedl, P., and S. Alexander. 2011. "Cancer invasion and the microenvironment: plasticity and reciprocity." *Cell* 147 (5):992-1009. doi: 10.1016/j.cell.2011.11.016.
Friedl, P., and E. B. Brocker. 2000. "The biology of cell locomotion within three-dimensional extracellular matrix." *Cell Mol Life Sci* 57 (1):41-64. doi: 10.1007/s000180050498.
Friedl, P., and K. Wolf. 2003. "Tumour-cell invasion and migration: diversity and escape mechanisms." *Nat Rev Cancer* 3 (5):362-74. doi: 10.1038/nrc1075.
Friedl, P., and K. Wolf. 2009. "Proteolytic interstitial cell migration: a five-step process." *Cancer Metastasis Rev* 28 (1-2):129-35. doi: 10.1007/s10555-008-9174-3.
Fujimoto-Ouchi, K., F. Sekiguchi, H. Yasuno, Y. Moriya, K. Mori, and Y. Tanaka. 2007. "Antitumor activity of trastuzumab in combination with chemotherapy in human gastric cancer xenograft models." *Cancer Chemother Pharmacol* 59 (6):795-805. doi: 10.1007/s00280-006-0337-z.
Fuse, N., Y. Kuboki, T. Kuwata, T. Nishina, S. Kadowaki, E. Shinozaki, N. Machida, S. Yuki, A. Ooki, S. Kajiura, T. Kimura, T. Yamanaka, K. Shitara, A. K. Nagatsuma, T. Yoshino, A. Ochiai, and A. Ohtsu. 2016. "Prognostic impact of HER2, EGFR, and c-MET status on overall survival of advanced gastric cancer patients." *Gastric Cancer* 19 (1):183-91. doi: 10.1007/s10120-015-0471-6.
Gajria, D., and S. Chandarlapaty. 2011. "HER2-amplified breast cancer: mechanisms of trastuzumab resistance and novel targeted therapies." *Expert Rev Anticancer Ther* 11 (2):263-75. doi: 10.1586/era.10.226.
Garcia, I., F. Vizoso, A. Martin, L. Sanz, O. Abdel-Lah, P. Raigoso, and J. L. Garcia-Muniz. 2003. "Clinical significance of the epidermal growth factor receptor and HER2 receptor in resectable gastric cancer." *Ann Surg Oncol* 10 (3):234-41.
Garner, A. P., C. U. Bialucha, E. R. Sprague, J. T. Garrett, Q. Sheng, S. Li, O. Sineshchekova, P. Saxena, C. R. Sutton, D. Chen, Y. Chen, H. Wang, J. Liang, R. Das, R. Mosher, J. Gu, A. Huang, N. Haubst, C. Zehetmeier, M. Haberl, W. Elis, C.

Kunz, A. B. Heidt, K. Herlihy, J. Murtie, A. Schuller, C. L. Arteaga, W. R. Sellers, and S. A. Ettenberg. 2013. "An antibody that locks HER3 in the inactive conformation inhibits tumor growth driven by HER2 or neuregulin." *Cancer Res* 73 (19):6024-35. doi: 10.1158/0008-5472.CAN-13-1198.

Garrett, J. T., M. G. Olivares, C. Rinehart, N. D. Granja-Ingram, V. Sanchez, A. Chakrabarty, B. Dave, R. S. Cook, W. Pao, E. McKinely, H. C. Manning, J. Chang, and C. L. Arteaga. 2011. "Transcriptional and posttranslational up-regulation of HER3 (ErbB3) compensates for inhibition of the HER2 tyrosine kinase." *Proc Natl Acad Sci U S A* 108 (12):5021-6. doi: 10.1073/pnas.1016140108.

Garrett, T. P., N. M. McKern, M. Lou, T. C. Elleman, T. E. Adams, G. O. Lovrecz, H. J. Zhu, F. Walker, M. J. Frenkel, P. A. Hoyne, R. N. Jorissen, E. C. Nice, A. W. Burgess, and C. W. Ward. 2002. "Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor alpha." *Cell* 110 (6):763-73.

Gay, C. M., K. Balaji, and L. A. Byers. 2017. "Giving AXL the axe: targeting AXL in human malignancy." *Br J Cancer* 116 (4):415-423. doi: 10.1038/bjc.2016.428.

Geyer, C. E., J. Forster, D. Lindquist, S. Chan, C. G. Romieu, T. Pienkowski, A. Jagiello-Gruszfeld, J. Crown, A. Chan, B. Kaufman, D. Skarlos, M. Campone, N. Davidson, M. Berger, C. Oliva, S. D. Rubin, S. Stein, and D. Cameron. 2006. "Lapatinib plus capecitabine for HER2-positive advanced breast cancer." *N Engl J Med* 355 (26):2733-43. doi: 10.1056/NEJMoa064320.

Ghosh, R., A. Narasanna, S. E. Wang, S. Liu, A. Chakrabarty, J. M. Balko, A. M. Gonzalez-Angulo, G. B. Mills, E. Penuel, J. Winslow, J. Sperinde, R. Dua, S. Pidaparthi, A. Mukherjee, K. Leitzel, W. J. Kostler, A. Lipton, M. Bates, and C. L. Arteaga. 2011. "Trastuzumab has preferential activity against breast cancers driven by HER2 homodimers." *Cancer Res* 71 (5):1871-82. doi: 10.1158/0008-5472.CAN-10-1872.

Gianni, L., T. Pienkowski, Y. H. Im, L. Roman, L. M. Tseng, M. C. Liu, A. Lluch, E. Staroslawska, J. de la Haba-Rodriguez, S. A. Im, J. L. Pedrini, B. Poirier, P. Morandi, V. Semiglazov, V. Srimuninnimit, G. Bianchi, T. Szado, J. Ratnayake, G. Ross, and P. Valagussa. 2012. "Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial." *Lancet Oncol* 13 (1):25-32. doi: 10.1016/S1470-2045(11)70336-9.

Giltnane, J. M., C. B. Moeder, R. L. Camp, and D. L. Rimm. 2009. "Quantitative multiplexed analysis of ErbB family coexpression for primary breast cancer prognosis in a large retrospective cohort." *Cancer* 115 (11):2400-9. doi: 10.1002/cncr.24277.

Givant-Horwitz, V., B. Davidson, and R. Reich. 2005. "Laminin-induced signaling in tumor cells." *Cancer Lett* 223 (1):1-10. doi: 10.1016/j.canlet.2004.08.030.

Gjerdrum, C., C. Tiron, T. Hoiby, I. Stefansson, H. Haugen, T. Sandal, K. Collett, S. Li, E. McCormack, B. T. Gjertsen, D. R. Micklem, L. A. Akslen, C. Glackin, and J. B. Lorens. 2010. "Axl is an essential epithelial-to-mesenchymal transition-induced regulator of breast cancer metastasis and patient survival." *Proc Natl Acad Sci U S A* 107 (3):1124-9. doi: 10.1073/pnas.0909333107.

Gong, J., A. Morishita, K. Kurokohchi, J. Tani, K. Kato, H. Miyoshi, H. Inoue, M. Kobayashi, S. Liu, M. Murota, A. Muramatsu, K. Izuishi, Y. Suzuki, H. Yoshida, N. Uchida, K. Deguchi, H. Iwama, I. Ishimaru, and T. Masaki. 2010. "Use of protein array to investigate receptor tyrosine kinases activated in gastric cancer." *Int J Oncol* 36 (1):101-6.

Gonzalez, C. A., N. Sala, and T. Rokkas. 2013. "Gastric cancer: epidemiologic aspects." *Helicobacter* 18 Suppl 1:34-8. doi: 10.1111/hel.12082.

Graus-Porta, D., R. R. Beerli, J. M. Daly, and N. E. Hynes. 1997. "ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling." *EMBO J* 16 (7):1647-55. doi: 10.1093/emboj/16.7.1647.

Gravalos, C., and A. Jimeno. 2008. "HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target." *Ann Oncol* 19 (9):1523-9. doi: 10.1093/annonc/mdn169.

Green, J., R. Nusse, and R. van Amerongen. 2014. "The role of Ryk and Ror receptor tyrosine kinases in Wnt signal transduction." *Cold Spring Harb Perspect Biol* 6 (2). doi: 10.1101/cshperspect.a009175.

Greulich, H., T. H. Chen, W. Feng, P. A. Janne, J. V. Alvarez, M. Zappaterra, S. E. Bulmer, D. A. Frank, W. C. Hahn, W. R. Sellers, and M. Meyerson. 2005. "Oncogenic transformation by inhibitor-sensitive and -resistant EGFR mutants." *PLoS Med* 2 (11):e313. doi: 10.1371/journal.pmed.0020313.

Groenendijk, F. H., and R. Bernards. 2014. "Drug resistance to targeted therapies: deja vu all over again." *Mol Oncol* 8 (6):1067-83. doi: 10.1016/j.molonc.2014.05.004.

Guggenheim, D. E., and M. A. Shah. 2013. "Gastric cancer epidemiology and risk factors." *J Surg Oncol* 107 (3):230-6. doi: 10.1002/jso.23262.

Guilford, P. J., J. B. Hopkins, W. M. Grady, S. D. Markowitz, J. Willis, H. Lynch, A. Rajput, G. L. Wiesner, N. M. Lindor, L. J. Burgart, T. T. Toro, D. Lee, J. M. Limacher, D. W. Shaw, M. P. Findlay, and A. E. Reeve. 1999. "E-cadherin germline mutations define an inherited cancer syndrome dominated by diffuse gastric cancer." *Hum Mutat* 14 (3):249-55. doi: 10.1002/(SICI)1098-1004(1999)14:3<249::AID-HUMU8>3.0.CO;2-9.

Guo, T., S. S. Lee, W. H. Ng, Y. Zhu, C. S. Gan, J. Zhu, H. Wang, S. Huang, S. K. Sze, and O. L. Kon. 2011. "Global molecular dysfunctions in gastric cancer revealed by an integrated analysis of the phosphoproteome and transcriptome." *Cell Mol Life Sci* 68 (11):1983-2002. doi: 10.1007/s00018-010-0545-x.

Gupta, G. P., and J. Massague. 2006. "Cancer metastasis: building a framework." *Cell* 127 (4):679-95. doi: 10.1016/j.cell.2006.11.001.

Han, S., Y. Meng, Q. Tong, G. Li, X. Zhang, Y. Chen, S. Hu, L. Zheng, W. Tan, H. Li, Y. Chen, G. Zhang, B. Li, and Y. Guo. 2014. "The ErbB2-targeting antibody trastuzumab and the small-molecule SRC inhibitor saracatinib synergistically inhibit ErbB2-overexpressing gastric cancer." *MAbs* 6 (2):403-8. doi: 10.4161/mabs.27443.

Hanahan, D., and J. Folkman. 1996. "Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis." *Cell* 86 (3):353-64.

Hanahan, D., and R. A. Weinberg. 2011. "Hallmarks of cancer: the next generation." *Cell* 144 (5):646-74. doi: 10.1016/j.cell.2011.02.013.

Hanahan, Douglas, and Robert A Weinberg. 2000. "The Hallmarks of Cancer." *Cell* 100 (1):57-70.

Harari, P. M., G. W. Allen, and J. A. Bonner. 2007. "Biology of interactions: antiepidermal growth factor receptor agents." *J Clin Oncol* 25 (26):4057-65. doi: 10.1200/JCO.2007.11.8984.

Harris, R. J., A. A. Murnane, S. L. Utter, K. L. Wagner, E. T. Cox, G. D. Polastri, J. C. Helder, and M. B. Sliwkowski. 1993. "Assessing genetic heterogeneity in production cell lines: detection by peptide mapping of a low level Tyr to Gln sequence variant in a recombinant antibody." *Biotechnology (N Y)* 11 (11):1293-7.

Hartmann-Petersen, R., P. S. Walmod, A. Berezin, V. Berezin, and E. Bock. 2000. "Individual cell motility studied by time-lapse video recording: influence of experimental conditions." *Cytometry* 40 (4):260-70.

Hata, A., N. Katakami, R. Kaji, S. Fujita, and Y. Imai. 2013. "Does T790M disappear? Successful gefitinib rechallenge after T790M disappearance in a patient with EGFR-mutant non-small-cell lung cancer." *J Thorac Oncol* 8 (3):e27-9. doi: 10.1097/JTO.0b013e318282e047.

Hayashi, M., M. Inokuchi, Y. Takagi, H. Yamada, K. Kojima, J. Kumagai, T. Kawano, and K. Sugihara. 2008. "High expression of HER3 is associated with a decreased survival in gastric cancer." *Clin Cancer Res* 14 (23):7843-9. doi: 10.1158/1078-0432.CCR-08-1064.

He, X. X., L. Ding, Y. Lin, M. Shu, J. M. Wen, and L. Xue. 2015. "Protein expression of HER2, 3, 4 in gastric cancer: correlation with clinical features and survival." *J Clin Pathol* 68 (5):374-80. doi: 10.1136/jclinpath-2014-202657.

Heindl, S., E. Eggenstein, S. Keller, J. Kneissl, G. Keller, K. Mutze, S. Rauser, G. Gasteiger, I. Drexler, A. Hapfelmeier, H. Hofler, and B. Luber. 2012. "Relevance of MET activation and genetic alterations of KRAS and E-cadherin for cetuximab sensitivity of gastric cancer cell lines." *J Cancer Res Clin Oncol* 138 (5):843-58. doi: 10.1007/s00432-011-1128-4.

Hellenic, Cooperative Oncology Group. 2017. Clinical trial of chemotherapy combination cisplatin-fluorouracilafatinib in patients with inoperable gastric cancer (AGAPP). https://ClinicalTrials.gov/show/NCT01743365.

Hennessy, B. T., D. L. Smith, P. T. Ram, Y. Lu, and G. B. Mills. 2005. "Exploiting the PI3K/AKT pathway for cancer drug discovery." *Nat Rev Drug Discov* 4 (12):988-1004. doi: 10.1038/nrd1902.

Herrero, R., J. Parsonnet, and E. R. Greenberg. 2014. "Prevention of gastric cancer." *JAMA* 312 (12):1197-8. doi: 10.1001/jama.2014.10498.

Holbro, T., G. Civenni, and N. E. Hynes. 2003. "The ErbB receptors and their role in cancer progression." *Exp Cell Res* 284 (1):99-110.

Holly, Stephan P, Mark K Larson, and Leslie V Parise. 2000. "Multiple Roles of Integrins in Cell Motility." *Exp. Cell Res.* 261:69-74.

Hu, B., N. El Hajj, S. Sittler, N. Lammert, R. Barnes, and A. Meloni-Ehrig. 2012. "Gastric cancer: Classification, histology and application of molecular pathology." *J Gastrointest Oncol* 3 (3):251-61. doi: 10.3978/j.issn.2078-6891.2012.021.

Huang, X., L. Gao, S. Wang, J. L. McManaman, A. D. Thor, X. Yang, F. J. Esteva, and B. Liu. 2010. "Heterotrimerization of the growth factor receptors erbB2, erbB3, and

insulin-like growth factor-i receptor in breast cancer cells resistant to herceptin." *Cancer Res* 70 (3):1204-14. doi: 10.1158/0008-5472.CAN-09-3321.
Hubbard, S. R. 2005. "EGF receptor inhibition: attacks on multiple fronts." *Cancer Cell* 7 (4):287-8. doi: 10.1016/j.ccr.2005.04.004.
Hudis, C. A. 2007. "Trastuzumab--mechanism of action and use in clinical practice." *N Engl J Med* 357 (1):39-51. doi: 10.1056/NEJMra043186.
Hynes, N. E., and H. A. Lane. 2005. "ERBB receptors and cancer: the complexity of targeted inhibitors." *Nat Rev Cancer* 5 (5):341-54. doi: 10.1038/nrc1609.
Hynes, R. O. 2002. "Integrins: bidirectional, allosteric signaling machines." *Cell* 110 (6):673-87.
Ishida, M., S. Kagawa, K. Shimoyama, K. Takehara, K. Noma, S. Tanabe, Y. Shirakawa, H. Tazawa, H. Kobayashi, and T. Fujiwara. 2016. "Trastuzumab-Based Photoimmunotherapy Integrated with Viral HER2 Transduction Inhibits Peritoneally Disseminated HER2-Negative Cancer." *Mol Cancer Ther* 15 (3):402-11. doi: 10.1158/1535-7163.MCT-15-0644.
Islam, S., C. M. Calkins, A. B. Goldin, C. Chen, C. D. Downard, E. Y. Huang, L. Cassidy, J. Saito, M. L. Blakely, S. J. Rangel, M. J. Arca, F. Abdullah, S. D. St Peter, Apsa Outcomes, and Committee Clinical Trials. 2012. "The diagnosis and management of empyema in children: a comprehensive review from the APSA Outcomes and Clinical Trials Committee." *J Pediatr Surg* 47 (11):2101-10. doi: 10.1016/j.jpedsurg.2012.07.047.
Izumi, Y., L. Xu, E. di Tomaso, D. Fukumura, and R. K. Jain. 2002. "Tumour biology: herceptin acts as an anti-angiogenic cocktail." *Nature* 416 (6878):279-80. doi: 10.1038/416279b.
Jackson, C. B., L. M. Judd, T. R. Menheniott, I. Kronborg, C. Dow, N. D. Yeomans, A. Boussioutas, L. Robb, and A. S. Giraud. 2007. "Augmented gp130-mediated cytokine signalling accompanies human gastric cancer progression." *J Pathol* 213 (2):140-51. doi: 10.1002/path.2218.
Jakszyn, P., and C. A. Gonzalez. 2006. "Nitrosamine and related food intake and gastric and oesophageal cancer risk: a systematic review of the epidemiological evidence." *World J Gastroenterol* 12 (27):4296-303.
Janjigian, Y. Y., N. Viola-Villegas, J. P. Holland, V. Divilov, S. D. Carlin, E. M. Gomes-DaGama, G. Chiosis, G. Carbonetti, E. de Stanchina, and J. S. Lewis. 2013. "Monitoring afatinib treatment in HER2-positive gastric cancer with 18F-FDG and 89Zr-trastuzumab PET." *J Nucl Med* 54 (6):936-43. doi: 10.2967/jnumed.112.110239.
Janjigian, Y. Y., D. Werner, C. Pauligk, K. Steinmetz, D. P. Kelsen, E. Jager, H. M. Altmannsberger, E. Robinson, L. J. Tafe, L. H. Tang, M. A. Shah, and S. E. Al-Batran. 2012. "Prognosis of metastatic gastric and gastroesophageal junction cancer by HER2 status: a European and USA International collaborative analysis." *Ann Oncol* 23 (10):2656-62. doi: 10.1093/annonc/mds104.
Ji, H., D. Li, L. Chen, T. Shimamura, S. Kobayashi, K. McNamara, U. Mahmood, A. Mitchell, Y. Sun, R. Al-Hashem, L. R. Chirieac, R. Padera, R. T. Bronson, W. Kim, P. A. Janne, G. I. Shapiro, D. Tenen, B. E. Johnson, R. Weissleder, N. E. Sharpless, and K. K. Wong. 2006. "The impact of human EGFR kinase domain

mutations on lung tumorigenesis and in vivo sensitivity to EGFR-targeted therapies." *Cancer Cell* 9 (6):485-95. doi: 10.1016/j.ccr.2006.04.022.

Johnson, E., D. D. Seachrist, C. M. DeLeon-Rodriguez, K. L. Lozada, J. Miedler, F. W. Abdul-Karim, and R. A. Keri. 2010. "HER2/ErbB2-induced breast cancer cell migration and invasion require p120 catenin activation of Rac1 and Cdc42." *J Biol Chem* 285 (38):29491-501. doi: 10.1074/jbc.M110.136770.

Jorissen, R. N., F. Walker, N. Pouliot, T. P. Garrett, C. W. Ward, and A. W. Burgess. 2003. "Epidermal growth factor receptor: mechanisms of activation and signalling." *Exp Cell Res* 284 (1):31-53.

Junior, P. N., R. A. Neto, and N. M. Forones. 2016. "Her2 Expression as a Prognostic Factor in Metastatic Gastric Cancer." *Arq Gastroenterol* 53 (2):62-7. doi: 10.1590/S0004-28032016000200003.

Junttila, T. T., R. W. Akita, K. Parsons, C. Fields, G. D. Lewis Phillips, L. S. Friedman, D. Sampath, and M. X. Sliwkowski. 2009. "Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941." *Cancer Cell* 15 (5):429-40. doi: 10.1016/j.ccr.2009.03.020.

Jura, N., Y. Shan, X. Cao, D. E. Shaw, and J. Kuriyan. 2009. "Structural analysis of the catalytically inactive kinase domain of the human EGF receptor 3." *Proc Natl Acad Sci U S A* 106 (51):21608-13. doi: 10.1073/pnas.0912101106.

Kakiuchi, M., T. Nishizawa, H. Ueda, K. Gotoh, A. Tanaka, A. Hayashi, S. Yamamoto, K. Tatsuno, H. Katoh, Y. Watanabe, T. Ichimura, T. Ushiku, S. Funahashi, K. Tateishi, I. Wada, N. Shimizu, S. Nomura, K. Koike, Y. Seto, M. Fukayama, H. Aburatani, and S. Ishikawa. 2014. "Recurrent gain-of-function mutations of RHOA in diffuse-type gastric carcinoma." *Nat Genet* 46 (6):583-7. doi: 10.1038/ng.2984.

Kang, S., A. G. Bader, and P. K. Vogt. 2005. "Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic." *Proc Natl Acad Sci U S A* 102 (3):802-7. doi: 10.1073/pnas.0408864102.

Kang, Y. K., W. K. Kang, D. B. Shin, J. Chen, J. Xiong, J. Wang, M. Lichinitser, Z. Guan, R. Khasanov, L. Zheng, M. Philco-Salas, T. Suarez, J. Santamaria, G. Forster, and P. I. McCloud. 2009. "Capecitabine/cisplatin versus 5-fluorouracil/cisplatin as first-line therapy in patients with advanced gastric cancer: a randomised phase III noninferiority trial." *Ann Oncol* 20 (4):666-73. doi: 10.1093/annonc/mdn717.

Kang, Y. K., S. Y. Rha, P. Tassone, J. Barriuso, R. Yu, T. Szado, A. Garg, and Y. J. Bang. 2014. "A phase IIa dose-finding and safety study of first-line pertuzumab in combination with trastuzumab, capecitabine and cisplatin in patients with HER2-positive advanced gastric cancer." *Br J Cancer* 111 (4):660-6. doi: 10.1038/bjc.2014.356.

Karakas, B., K. E. Bachman, and B. H. Park. 2006. "Mutation of the PIK3CA oncogene in human cancers." *Br J Cancer* 94 (4):455-9. doi: 10.1038/sj.bjc.6602970.

Kassis, J., D. A. Lauffenburger, T. Turner, and A. Wells. 2001. "Tumor invasion as dysregulated cell motility." *Semin Cancer Biol* 11 (2):105-17. doi: 10.1006/scbi.2000.0362.

Kawai, S., S. Kato, H. Imai, Y. Okada, and C. Ishioka. 2013. "Suppression of FUT1 attenuates cell proliferation in the HER2-overexpressing cancer cell line NCI-N87." *Oncol Rep* 29 (1):13-20. doi: 10.3892/or.2012.2120.

Keller, S.*, G.* Zwingenberger, K. Ebert, J. Hasenauer, J. Wasmuth, D. Maier, I. Haffner, K. Schierle, G. Weirich, and B. Luber. 2018. "Effects of trastuzumab and afatinib on kinase activity in gastric cancer cell lines." *Mol Oncol* 12 (4):441-462. doi: 10.1002/1878-0261.12170.

Kelley, J. R., and J. M. Duggan. 2003. "Gastric cancer epidemiology and risk factors." *J Clin Epidemiol* 56 (1):1-9.

Kesarwala, A. H., M. M. Samrakandi, and D. Piwnica-Worms. 2009. "Proteasome inhibition blocks ligand-induced dynamic processing and internalization of epidermal growth factor receptor via altered receptor ubiquitination and phosphorylation." *Cancer Res* 69 (3):976-83. doi: 10.1158/0008-5472.CAN-08-2938.

Khoury, H., M. A. Naujokas, D. Zuo, V. Sangwan, M. M. Frigault, S. Petkiewicz, D. L. Dankort, W. J. Muller, and M. Park. 2005. "HGF converts ErbB2/Neu epithelial morphogenesis to cell invasion." *Mol Biol Cell* 16 (2):550-61. doi: 10.1091/mbc.E04-07-0567.

Kim, H. H., S. L. Sierke, and J. G. Koland. 1994. "Epidermal growth factor-dependent association of phosphatidylinositol 3-kinase with the erbB3 gene product." *J Biol Chem* 269 (40):24747-55.

Kim, I. J., J. H. Park, H. C. Kang, Y. Shin, H. W. Park, H. R. Park, J. L. Ku, S. B. Lim, and J. G. Park. 2003. "Mutational analysis of BRAF and K-ras in gastric cancers: absence of BRAF mutations in gastric cancers." *Hum Genet* 114 (1):118-20. doi: 10.1007/s00439-003-1027-0.

Kim, J. W., H. P. Kim, S. A. Im, S. Kang, H. S. Hur, Y. K. Yoon, D. Y. Oh, J. H. Kim, D. S. Lee, T. Y. Kim, and Y. J. Bang. 2008. "The growth inhibitory effect of lapatinib, a dual inhibitor of EGFR and HER2 tyrosine kinase, in gastric cancer cell lines." *Cancer Lett* 272 (2):296-306. doi: 10.1016/j.canlet.2008.07.018.

Kim, M. A., E. J. Jung, H. S. Lee, H. E. Lee, Y. K. Jeon, H. K. Yang, and W. H. Kim. 2007. "Evaluation of HER-2 gene status in gastric carcinoma using immunohistochemistry, fluorescence in situ hybridization, and real-time quantitative polymerase chain reaction." *Hum Pathol* 38 (9):1386-93. doi: 10.1016/j.humpath.2007.02.005.

Kim, M. A., H. S. Lee, H. E. Lee, Y. K. Jeon, H. K. Yang, and W. H. Kim. 2008. "EGFR in gastric carcinomas: prognostic significance of protein overexpression and high gene copy number." *Histopathology* 52 (6):738-46. doi: 10.1111/j.1365-2559.2008.03021.x.

Kim, S. Y., H. P. Kim, Y. J. Kim, Y. Oh do, S. A. Im, D. Lee, H. S. Jong, T. Y. Kim, and Y. J. Bang. 2008. "Trastuzumab inhibits the growth of human gastric cancer cell lines with HER2 amplification synergistically with cisplatin." *Int J Oncol* 32 (1):89-95.

Kimura, H., K. Sakai, T. Arao, T. Shimoyama, T. Tamura, and K. Nishio. 2007. "Antibody-dependent cellular cytotoxicity of cetuximab against tumor cells with wild-type or mutant epidermal growth factor receptor." *Cancer Sci* 98 (8):1275-80. doi: 10.1111/j.1349-7006.2007.00510.x.

Kinzler, K. W., and B. Vogelstein. 1996. "Lessons from hereditary colorectal cancer." *Cell* 87 (2):159-70.

Klapper, L. N., H. Waterman, M. Sela, and Y. Yarden. 2000. "Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2." *Cancer Res* 60 (13):3384-8.

Klos, K. S., X. Zhou, S. Lee, L. Zhang, W. Yang, Y. Nagata, and D. Yu. 2003. "Combined trastuzumab and paclitaxel treatment better inhibits ErbB-2-mediated angiogenesis in breast carcinoma through a more effective inhibition of Akt than either treatment alone." *Cancer* 98 (7):1377-85. doi: 10.1002/cncr.11656.

Kneissl, J., A. Hartmann, N. Pfarr, F. Erlmeier, T. Lorber, S. Keller, G. Zwingenberger, W. Weichert, and B. Luber. 2017. "Influence of the HER receptor ligand system on sensitivity to cetuximab and trastuzumab in gastric cancer cell lines." *J Cancer Res Clin Oncol* 143 (4):573-600. doi: 10.1007/s00432-016-2308-z.

Kneissl, J., S. Keller, T. Lorber, S. Heindl, G. Keller, I. Drexler, A. Hapfelmeier, H. Hofler, and B. Luber. 2012. "Association of amphiregulin with the cetuximab sensitivity of gastric cancer cell lines." *Int J Oncol* 41 (2):733-44. doi: 10.3892/ijo.2012.1479.

Knudson, A. G., Jr. 1979. "Mutagenesis and embryonal carcinogenesis." *Natl Cancer Inst Monogr* (51):19-24.

Ko, B. K., S. Y. Lee, Y. H. Lee, I. S. Hwang, H. Persson, J. Rockberg, C. Borrebaeck, D. Park, K. T. Kim, M. Uhlen, and J. S. Lee. 2015. "Combination of novel HER2-targeting antibody 1E11 with trastuzumab shows synergistic antitumor activity in HER2-positive gastric cancer." *Mol Oncol* 9 (2):398-408. doi: 10.1016/j.molonc.2014.09.007.

Koenig, A., C. Mueller, C. Hasel, G. Adler, and A. Menke. 2006. "Collagen type I induces disruption of E-cadherin-mediated cell-cell contacts and promotes proliferation of pancreatic carcinoma cells." *Cancer Res* 66 (9):4662-71. doi: 10.1158/0008-5472.CAN-05-2804.

Kol, A., A. G. Terwisscha van Scheltinga, H. Timmer-Bosscha, L. E. Lamberts, F. Bensch, E. G. de Vries, and C. P. Schroder. 2014. "HER3, serious partner in crime: therapeutic approaches and potential biomarkers for effect of HER3-targeting." *Pharmacol Ther* 143 (1):1-11. doi: 10.1016/j.pharmthera.2014.01.005.

Konecny, G. E., M. D. Pegram, N. Venkatesan, R. Finn, G. Yang, M. Rahmeh, M. Untch, D. W. Rusnak, G. Spehar, R. J. Mullin, B. R. Keith, T. M. Gilmer, M. Berger, K. C. Podratz, and D. J. Slamon. 2006. "Activity of the dual kinase inhibitor lapatinib (GW572016) against HER-2-overexpressing and trastuzumab-treated breast cancer cells." *Cancer Res* 66 (3):1630-9. doi: 10.1158/0008-5472.CAN-05-1182.

Korshunov, V. A. 2012. "Axl-dependent signalling: a clinical update." *Clin Sci (Lond)* 122 (8):361-8. doi: 10.1042/CS20110411.

Kostler, W. J., B. Schwab, C. F. Singer, R. Neumann, E. Rucklinger, T. Brodowicz, S. Tomek, M. Niedermayr, M. Hejna, G. G. Steger, M. Krainer, C. Wiltschke, and C. C. Zielinski. 2004. "Monitoring of serum Her-2/neu predicts response and progression-free survival to trastuzumab-based treatment in patients with metastatic breast cancer." *Clin Cancer Res* 10 (5):1618-24.

Krams, Matthias, Sven Olaf Frahm, Udo Kellner, and Christian Mawrin. 2013. *Kurzlehrbuch Pathologie*. Edited by aktual. Aufl. 2. Stuttgart: Thieme.

Kurreck, J. 2009. "RNA interference: from basic research to therapeutic applications." *Angew Chem Int Ed Engl* 48 (8):1378-98. doi: 10.1002/anie.200802092.

Kwon, Y. K., A. Bhattacharyya, J. A. Alberta, W. V. Giannobile, K. Cheon, C. D. Stiles, and S. L. Pomeroy. 1997. "Activation of ErbB2 during wallerian degeneration of sciatic nerve." *J Neurosci* 17 (21):8293-9.

Kwong, K. Y., and M. C. Hung. 1998. "A novel splice variant of HER2 with increased transformation activity." *Mol Carcinog* 23 (2):62-8.

La Vecchia, C., E. Negri, S. Franceschi, and A. Gentile. 1992. "Family history and the risk of stomach and colorectal cancer." *Cancer* 70 (1):50-5.

Lamalice, L., F. Le Boeuf, and J. Huot. 2007. "Endothelial cell migration during angiogenesis." *Circ Res* 100 (6):782-94. doi: 10.1161/01.RES.0000259593.07661.1e.

Lane, H. A., I. Beuvink, A. B. Motoyama, J. M. Daly, R. M. Neve, and N. E. Hynes. 2000. "ErbB2 potentiates breast tumor proliferation through modulation of p27(Kip1)-Cdk2 complex formation: receptor overexpression does not determine growth dependency." *Mol Cell Biol* 20 (9):3210-23.

Lane, H. A., A. B. Motoyama, I. Beuvink, and N. E. Hynes. 2001. "Modulation of p27/Cdk2 complex formation through 4D5-mediated inhibition of HER2 receptor signaling." *Ann Oncol* 12 Suppl 1:S21-2.

Lauren, P. 1965. "The Two Histological Main Types of Gastric Carcinoma: Diffuse and So-Called Intestinal-Type Carcinoma. An Attempt at a Histo-Clinical Classification." *Acta Pathol Microbiol Scand* 64:31-49.

Leavesley, D. I., G. D. Ferguson, E. A. Wayner, and D. A. Cheresh. 1992. "Requirement of the integrin beta 3 subunit for carcinoma cell spreading or migration on vitronectin and fibrinogen." *J Cell Biol* 117 (5):1101-7.

Lee, J. W., Y. H. Soung, S. H. Seo, S. Y. Kim, C. H. Park, Y. P. Wang, K. Park, S. W. Nam, W. S. Park, S. H. Kim, J. Y. Lee, N. J. Yoo, and S. H. Lee. 2006. "Somatic mutations of ERBB2 kinase domain in gastric, colorectal, and breast carcinomas." *Clin Cancer Res* 12 (1):57-61. doi: 10.1158/1078-0432.CCR-05-0976.

Leitzel, K., Y. Teramoto, K. Konrad, V. M. Chinchilli, G. Volas, H. Grossberg, H. Harvey, L. Demers, and A. Lipton. 1995. "Elevated serum c-erbB-2 antigen levels and decreased response to hormone therapy of breast cancer." *J Clin Oncol* 13 (5):1129-35.

Lemoine, N. R., S. Jain, F. Silvestre, C. Lopes, C. M. Hughes, E. McLelland, W. J. Gullick, and M. I. Filipe. 1991. "Amplification and overexpression of the EGF receptor and c-erbB-2 proto-oncogenes in human stomach cancer." *Br J Cancer* 64 (1):79-83.

Leto, S. M., F. Sassi, I. Catalano, V. Torri, G. Migliardi, E. R. Zanella, M. Throsby, A. Bertotti, and L. Trusolino. 2015. "Sustained Inhibition of HER3 and EGFR Is Necessary to Induce Regression of HER2-Amplified Gastrointestinal Carcinomas." *Clin Cancer Res* 21 (24):5519-31. doi: 10.1158/1078-0432.CCR-14-3066.

Li, A., T. Zhou, L. Guo, and J. Si. 2010. "Collagen type I regulates beta-catenin tyrosine phosphorylation and nuclear translocation to promote migration and proliferation of gastric carcinoma cells." *Oncol Rep* 23 (5):1247-55.

Li, D., L. Ambrogio, T. Shimamura, S. Kubo, M. Takahashi, L. R. Chirieac, R. F. Padera, G. I. Shapiro, A. Baum, F. Himmelsbach, W. J. Rettig, M. Meyerson, F. Solca, H. Greulich, and K. K. Wong. 2008. "BIBW2992, an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models." *Oncogene* 27 (34):4702-11. doi: 10.1038/onc.2008.109.

Li, S., K. R. Schmitz, P. D. Jeffrey, J. J. Wiltzius, P. Kussie, and K. M. Ferguson. 2005. "Structural basis for inhibition of the epidermal growth factor receptor by cetuximab." *Cancer Cell* 7 (4):301-11. doi: 10.1016/j.ccr.2005.03.003.

Lin, Y. Z., and G. M. Clinton. 1991. "A soluble protein related to the HER-2 proto-oncogene product is released from human breast carcinoma cells." *Oncogene* 6 (4):639-43.

Liu, E., B. Hjelle, and J. M. Bishop. 1988. "Transforming genes in chronic myelogenous leukemia." *Proc Natl Acad Sci U S A* 85 (6):1952-6.

Liu, L., J. Greger, H. Shi, Y. Liu, J. Greshock, R. Annan, W. Halsey, G. M. Sathe, A. M. Martin, and T. M. Gilmer. 2009. "Novel mechanism of lapatinib resistance in HER2-positive breast tumor cells: activation of AXL." *Cancer Res* 69 (17):6871-8. doi: 10.1158/0008-5472.CAN-08-4490.

Liu, L., H. Shi, Y. Liu, A. Anderson, J. Peterson, J. Greger, A. M. Martin, and T. M. Gilmer. 2011. "Synergistic effects of foretinib with HER-targeted agents in MET and HER1- or HER2-coactivated tumor cells." *Mol Cancer Ther* 10 (3):518-30. doi: 10.1158/1535-7163.MCT-10-0698.

Liu, S. D., C. Chalouni, J. C. Young, T. T. Junttila, M. X. Sliwkowski, and J. B. Lowe. 2015. "Afucosylated antibodies increase activation of FcgammaRIIIa-dependent signaling components to intensify processes promoting ADCC." *Cancer Immunol Res* 3 (2):173-83. doi: 10.1158/2326-6066.CIR-14-0125.

Lordick, F., W. Allum, F. Carneiro, E. Mitry, J. Tabernero, P. Tan, E. Van Cutsem, C. van de Velde, and A. Cervantes. 2014. "Unmet needs and challenges in gastric cancer: the way forward." *Cancer Treat Rev* 40 (6):692-700. doi: 10.1016/j.ctrv.2014.03.002.

Lordick, F., Y. K. Kang, H. C. Chung, P. Salman, S. C. Oh, G. Bodoky, G. Kurteva, C. Volovat, V. M. Moiseyenko, V. Gorbunova, J. O. Park, A. Sawaki, I. Celik, H. Gotte, H. Melezinkova, M. Moehler, Onkologie Arbeitsgemeinschaft Internistische, and Expand Investigators. 2013. "Capecitabine and cisplatin with or without cetuximab for patients with previously untreated advanced gastric cancer (EXPAND): a randomised, open-label phase 3 trial." *Lancet Oncol* 14 (6):490-9. doi: 10.1016/S1470-2045(13)70102-5.

Lordick, F., M Knödler, G. Stocker, I Haffner, U Hacker, A Hoffmeister, and J Mössner. 2015. "Moderne Pharmakologische Therapiekonzepte." *Onkologie heute* (04):5.

Lorenzen, S., J. Riera Knorrenschild, G. M. Haag, M. Pohl, P. Thuss-Patience, F. Bassermann, U. Helbig, F. Weissinger, E. Schnoy, K. Becker, G. Stocker, J. Ruschoff, A. Eisenmenger, I. Karapanagiotou-Schenkel, and F. Lordick. 2015. "Lapatinib versus lapatinib plus capecitabine as second-line treatment in human epidermal growth factor receptor 2-amplified metastatic gastro-oesophageal cancer: a randomised phase II trial of the Arbeitsgemeinschaft Internistische Onkologie." *Eur J Cancer* 51 (5):569-76. doi: 10.1016/j.ejca.2015.01.059.

Lu, Y., X. Zi, Y. Zhao, D. Mascarenhas, and M. Pollak. 2001. "Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin)." *J Natl Cancer Inst* 93 (24):1852-7.

Lynch, H. T., W. Grady, G. Suriano, and D. Huntsman. 2005. "Gastric cancer: new genetic developments." *J Surg Oncol* 90 (3):114-33; discussion 133. doi: 10.1002/jso.20214.

Mahadevan, D., L. Cooke, C. Riley, R. Swart, B. Simons, K. Della Croce, L. Wisner, M. Iorio, K. Shakalya, H. Garewal, R. Nagle, and D. Bearss. 2007. "A novel tyrosine kinase switch is a mechanism of imatinib resistance in gastrointestinal stromal tumors." *Oncogene* 26 (27):3909-19. doi: 10.1038/sj.onc.1210173.

Majewski, I. J., P. Nuciforo, L. Mittempergher, A. J. Bosma, H. Eidtmann, E. Holmes, C. Sotiriou, D. Fumagalli, J. Jimenez, C. Aura, L. Prudkin, M. C. Diaz-Delgado, L. de la Pena, S. Loi, C. Ellis, N. Schultz, E. de Azambuja, N. Harbeck, M. Piccart-Gebhart, R. Bernards, and J. Baselga. 2015. "PIK3CA mutations are associated with decreased benefit to neoadjuvant human epidermal growth factor receptor 2-targeted therapies in breast cancer." *J Clin Oncol* 33 (12):1334-9. doi: 10.1200/JCO.2014.55.2158.

Marais, R., Y. Light, H. F. Paterson, and C. J. Marshall. 1995. "Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation." *EMBO J* 14 (13):3136-45.

Marchini, C, L Pietrella, C Kalogris, C Garulli, F Gabrielli, E Quaglino, M Iezzi, S.M. Pupa, E Tagliabue, and A Amici. 2013. *HER2-Driven Carcinogenesis: New Mouse Models for Novel Immunotherapies, Oncogene and Cancer - From Bench to Clinic*. Edited by Dr. Yahwardiah Siregar (Ed.): InTech.

Marmor, M. D., K. B. Skaria, and Y. Yarden. 2004. "Signal transduction and oncogenesis by ErbB/HER receptors." *Int J Radiat Oncol Biol Phys* 58 (3):903-13. doi: 10.1016/j.ijrobp.2003.06.002.

Marshall, B. J., and J. R. Warren. 1984. "Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration." *Lancet* 1 (8390):1311-5.

Massague, J., and A. Pandiella. 1993. "Membrane-anchored growth factors." *Annu Rev Biochem* 62:515-41. doi: 10.1146/annurev.bi.62.070193.002503.

Matsui, Y., M. Inomata, M. Tojigamori, K. Sonoda, N. Shiraishi, and S. Kitano. 2005. "Suppression of tumor growth in human gastric cancer with HER2 overexpression by an anti-HER2 antibody in a murine model." *Int J Oncol* 27 (3):681-5.

Memorial Sloan Kettering Cancer Center. 2017. "Afatinib and Paclitaxel in Patients With Advanced HER2-Positive Trastuzumab-Refractory Advanced Esophagogastric Cancer." *ClinicalTrials.gov [Internet]. Bethesda (MD): National Library of Medicine (US). Available from https://ClinicalTrials.gov/show/NCT01522768*.

Menke, A., C. Philippi, R. Vogelmann, B. Seidel, M. P. Lutz, G. Adler, and D. Wedlich. 2001. "Down-regulation of E-cadherin gene expression by collagen type I and type III in pancreatic cancer cell lines." *Cancer Res* 61 (8):3508-17.

Meriggi, F., W. Vermi, P. Bertocchi, and A. Zaniboni. 2014. "The Emerging Role of NRAS Mutations in Colorectal Cancer Patients Selected for Anti-EGFR Therapies." *Rev Recent Clin Trials* 9 (1):8-12.

Meyer, A. S., M. A. Miller, F. B. Gertler, and D. A. Lauffenburger. 2013. "The receptor AXL diversifies EGFR signaling and limits the response to EGFR-targeted inhibitors in triple-negative breast cancer cells." *Sci Signal* 6 (287):ra66. doi: 10.1126/scisignal.2004155.

Miettinen, P. J., J. E. Berger, J. Meneses, Y. Phung, R. A. Pedersen, Z. Werb, and R. Derynck. 1995. "Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor." *Nature* 376 (6538):337-41. doi: 10.1038/376337a0.

Miller, M. A., R. J. Sullivan, and D. A. Lauffenburger. 2017. "Molecular Pathways: Receptor Ectodomain Shedding in Treatment, Resistance, and Monitoring of Cancer." *Clin Cancer Res* 23 (3):623-629. doi: 10.1158/1078-0432.CCR-16-0869.

Minuti, G., F. Cappuzzo, R. Duchnowska, J. Jassem, A. Fabi, T. O'Brien, A. D. Mendoza, L. Landi, W. Biernat, B. Czartoryska-Arlukowicz, T. Jankowski, D. Zuziak, J. Zok, B. Szostakiewicz, M. Foszczynska-Kloda, A. Tempinska-Szalach, E. Rossi, and M. Varella-Garcia. 2012. "Increased MET and HGF gene copy numbers are associated with trastuzumab failure in HER2-positive metastatic breast cancer." *Br J Cancer* 107 (5):793-9. doi: 10.1038/bjc.2012.335.

Mitra, D., M. J. Brumlik, S. U. Okamgba, Y. Zhu, T. T. Duplessis, J. G. Parvani, S. M. Lesko, E. Brogi, and F. E. Jones. 2009. "An oncogenic isoform of HER2 associated with locally disseminated breast cancer and trastuzumab resistance." *Mol Cancer Ther* 8 (8):2152-62. doi: 10.1158/1535-7163.MCT-09-0295.

Miyamoto, S., Y. Nagamura, A. Nakabo, A. Okabe, K. Yanagihara, K. Fukami, R. Sakai, and H. Yamaguchi. 2018. "Aberrant alternative splicing of RHOA is associated with loss of its expression and activity in diffuse-type gastric carcinoma cells." *Biochem Biophys Res Commun* 495 (2):1942-1947. doi: 10.1016/j.bbrc.2017.12.067.

Molina, M. A., J. Codony-Servat, J. Albanell, F. Rojo, J. Arribas, and J. Baselga. 2001. "Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells." *Cancer Res* 61 (12):4744-9.

Molina, M. A., R. Saez, E. E. Ramsey, M. J. Garcia-Barchino, F. Rojo, A. J. Evans, J. Albanell, E. J. Keenan, A. Lluch, J. Garcia-Conde, J. Baselga, and G. M. Clinton. 2002. "NH(2)-terminal truncated HER-2 protein but not full-length receptor is associated with nodal metastasis in human breast cancer." *Clin Cancer Res* 8 (2):347-53.

Montero, J. C., R. Rodriguez-Barrueco, A. Ocana, E. Diaz-Rodriguez, A. Esparis-Ogando, and A. Pandiella. 2008. "Neuregulins and cancer." *Clin Cancer Res* 14 (11):3237-41. doi: 10.1158/1078-0432.CCR-07-5133.

Motoyama, A. B., N. E. Hynes, and H. A. Lane. 2002. "The efficacy of ErbB receptor-targeted anticancer therapeutics is influenced by the availability of epidermal growth factor-related peptides." *Cancer Res* 62 (11):3151-8.

Motoyama, T., H. Hojo, and H. Watanabe. 1986. "Comparison of seven cell lines derived from human gastric carcinomas." *Acta Pathol Jpn* 36 (1):65-83.

Moya-Horno, I., and J. Cortes. 2015. "The expanding role of pertuzumab in the treatment of HER2-positive breast cancer." *Breast Cancer (Dove Med Press)* 7:125-32. doi: 10.2147/BCTT.S61579.

MSKCC, Memorial Sloan Kettering Cancer Center. 2017. Afatinib and paclitaxel in patients with advanced HER2-positive trastuzumab-refractory advanced esophagogastric cancer. https://ClinicalTrials.gov/show/NCT01522768.

Munoz, N., and S. Franceschi. 1997. "Epidemiology of gastric cancer and perspectives for prevention." *Salud Publica Mex* 39 (4):318-30.

Nagata, Y, KH Lan, X Zhou, M Tan, FJ Esteva, and AA Sahin. 2004. "PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients." *Cancer Cell* 6:117-127.

Nahta, R., and F. J. Esteva. 2006. "HER2 therapy: molecular mechanisms of trastuzumab resistance." *Breast Cancer Res* 8 (6):215. doi: 10.1186/bcr1612.

Nahta, R., L. X. Yuan, Y. Du, and F. J. Esteva. 2007. "Lapatinib induces apoptosis in trastuzumab-resistant breast cancer cells: effects on insulin-like growth factor I signaling." *Mol Cancer Ther* 6 (2):667-74. doi: 10.1158/1535-7163.MCT-06-0423.

Nam, H. J., K. A. Ching, J. Kan, H. P. Kim, S. W. Han, S. A. Im, T. Y. Kim, J. G. Christensen, D. Y. Oh, and Y. J. Bang. 2012. "Evaluation of the antitumor effects and mechanisms of PF00299804, a pan-HER inhibitor, alone or in combination with chemotherapy or targeted agents in gastric cancer." *Mol Cancer Ther* 11 (2):439-51. doi: 10.1158/1535-7163.MCT-11-0494.

Narayan, M., J. A. Wilken, L. N. Harris, A. T. Baron, K. D. Kimbler, and N. J. Maihle. 2009. "Trastuzumab-induced HER reprogramming in "resistant" breast carcinoma cells." *Cancer Res* 69 (6):2191-4. doi: 10.1158/0008-5472.CAN-08-1056.

Neubauer, A., A. Fiebeler, D. K. Graham, J. P. O'Bryan, C. A. Schmidt, P. Barckow, S. Serke, W. Siegert, H. R. Snodgrass, D. Huhn, and et al. 1994. "Expression of axl, a transforming receptor tyrosine kinase, in normal and malignant hematopoiesis." *Blood* 84 (6):1931-41.

Neve, R. M., H. A. Lane, and N. E. Hynes. 2001. "The role of overexpressed HER2 in transformation." *Ann Oncol* 12 Suppl 1:S9-13.

Niederst, M. J., and J. A. Engelman. 2013. "Bypass mechanisms of resistance to receptor tyrosine kinase inhibition in lung cancer." *Sci Signal* 6 (294):re6. doi: 10.1126/scisignal.2004652.

Nishikawa, R., X. D. Ji, R. C. Harmon, C. S. Lazar, G. N. Gill, W. K. Cavenee, and H. J. Huang. 1994. "A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity." *Proc Natl Acad Sci U S A* 91 (16):7727-31.

Nishinaka, T., and C. Yabe-Nishimura. 2001. "EGF receptor-ERK pathway is the major signaling pathway that mediates upregulation of aldose reductase expression under oxidative stress." *Free Radic Biol Med* 31 (2):205-16.

Nollet, Friedel, Patrick Kools, and Frans van Roy. 2000. "Phylogenetic Analysis of the Cadherin Superfamily allows Identification of Six Major Subfamilies Besides Several Solitary Members." *J. Mol. Biol.* 299:551-572.

Normanno, N., A. De Luca, C. Bianco, L. Strizzi, M. Mancino, M. R. Maiello, A. Carotenuto, G. De Feo, F. Caponigro, and D. S. Salomon. 2006. "Epidermal growth factor receptor (EGFR) signaling in cancer." *Gene* 366 (1):2-16. doi: 10.1016/j.gene.2005.10.018.

Nyati, M. K., M. A. Morgan, F. Y. Feng, and T. S. Lawrence. 2006. "Integration of EGFR inhibitors with radiochemotherapy." *Nat Rev Cancer* 6 (11):876-85. doi: 10.1038/nrc1953.

O'Bryan, J. P., R. A. Frye, P. C. Cogswell, A. Neubauer, B. Kitch, C. Prokop, R. Espinosa, 3rd, M. M. Le Beau, H. S. Earp, and E. T. Liu. 1991. "axl, a transforming gene isolated from primary human myeloid leukemia cells, encodes a novel receptor tyrosine kinase." *Mol Cell Biol* 11 (10):5016-31.

O'Donnell, K., I. C. Harkes, L. Dougherty, and I. P. Wicks. 1999. "Expression of receptor tyrosine kinase Axl and its ligand Gas6 in rheumatoid arthritis: evidence for a novel

endothelial cell survival pathway." *Am J Pathol* 154 (4):1171-80. doi: 10.1016/S0002-9440(10)65369-2.

Oda, T., Y. Kanai, T. Oyama, K. Yoshiura, Y. Shimoyama, W. Birchmeier, T. Sugimura, and S. Hirohashi. 1994. "E-cadherin gene mutations in human gastric carcinoma cell lines." *Proc Natl Acad Sci U S A* 91 (5):1858-62.

Ogiso, H., R. Ishitani, O. Nureki, S. Fukai, M. Yamanaka, J. H. Kim, K. Saito, A. Sakamoto, M. Inoue, M. Shirouzu, and S. Yokoyama. 2002. "Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains." *Cell* 110 (6):775-87.

Ohta, T., and M. Fukuda. 2004. "Ubiquitin and breast cancer." *Oncogene* 23 (11):2079-88. doi: 10.1038/sj.onc.1207371.

Okines, A., D. Cunningham, and I. Chau. 2011. "Targeting the human EGFR family in esophagogastric cancer." *Nat Rev Clin Oncol* 8 (8):492-503. doi: 10.1038/nrclinonc.2011.45.

Okines, A. F., and D. Cunningham. 2012. "Trastuzumab: a novel standard option for patients with HER-2-positive advanced gastric or gastro-oesophageal junction cancer." *Therap Adv Gastroenterol* 5 (5):301-18. doi: 10.1177/1756283X12450246.

Okines, A., M. Verheij, W. Allum, D. Cunningham, A. Cervantes, and Esmo Guidelines Working Group. 2010. "Gastric cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up." *Ann Oncol* 21 Suppl 5:v50-4. doi: 10.1093/annonc/mdq164.

Olayioye, M. A., R. M. Neve, H. A. Lane, and N. E. Hynes. 2000. "The ErbB signaling network: receptor heterodimerization in development and cancer." *EMBO J* 19 (13):3159-67. doi: 10.1093/emboj/19.13.3159.

Oliveira, C., H. Pinheiro, J. Figueiredo, R. Seruca, and F. Carneiro. 2013. "E-cadherin alterations in hereditary disorders with emphasis on hereditary diffuse gastric cancer." *Prog Mol Biol Transl Sci* 116:337-59. doi: 10.1016/B978-0-12-394311-8.00015-7.

Organ, S. L., and M. S. Tsao. 2011. "An overview of the c-MET signaling pathway." *Ther Adv Med Oncol* 3 (1 Suppl):S7-S19. doi: 10.1177/1758834011422556.

Ou, W. B., J. M. Corson, D. L. Flynn, W. P. Lu, S. C. Wise, R. Bueno, D. J. Sugarbaker, and J. A. Fletcher. 2011. "AXL regulates mesothelioma proliferation and invasiveness." *Oncogene* 30 (14):1643-52. doi: 10.1038/onc.2010.555.

Paccez, J. D., G. J. Vasques, R. G. Correa, J. F. Vasconcellos, K. Duncan, X. Gu, M. Bhasin, T. A. Libermann, and L. F. Zerbini. 2013. "The receptor tyrosine kinase Axl is an essential regulator of prostate cancer proliferation and tumor growth and represents a new therapeutic target." *Oncogene* 32 (6):689-98. doi: 10.1038/onc.2012.89.

Pao, W., V. A. Miller, K. A. Politi, G. J. Riely, R. Somwar, M. F. Zakowski, M. G. Kris, and H. Varmus. 2005. "Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain." *PLoS Med* 2 (3):e73. doi: 10.1371/journal.pmed.0020073.

Park, J. G., H. Frucht, R. V. LaRocca, D. P. Bliss, Jr., Y. Kurita, T. R. Chen, J. G. Henslee, J. B. Trepel, R. T. Jensen, B. E. Johnson, and et al. 1990. "Characteristics of cell lines established from human gastric carcinoma." *Cancer Res* 50 (9):2773-80.

Parkin, D. M. 2006. "The global health burden of infection-associated cancers in the year 2002." *Int J Cancer* 118 (12):3030-44. doi: 10.1002/ijc.21731.

Patel, D., R. Bassi, A. Hooper, M. Prewett, D. J. Hicklin, and X. Kang. 2009. "Anti-epidermal growth factor receptor monoclonal antibody cetuximab inhibits EGFR/HER-2 heterodimerization and activation." *Int J Oncol* 34 (1):25-32.

Piazuelo, M. B., and P. Correa. 2013. "Gastric cancer: Overview." *Colomb Med (Cali)* 44 (3):192-201.

Pitot, H. C. 1993. "The molecular biology of carcinogenesis." *Cancer* 72 (3 Suppl):962-70.

Politi, K., M. F. Zakowski, P. D. Fan, E. A. Schonfeld, W. Pao, and H. E. Varmus. 2006. "Lung adenocarcinomas induced in mice by mutant EGF receptors found in human lung cancers respond to a tyrosine kinase inhibitor or to down-regulation of the receptors." *Genes Dev* 20 (11):1496-510. doi: 10.1101/gad.1417406.

Polkowski, W., J. W. van Sandick, G. J. Offerhaus, F. J. ten Kate, J. Mulder, H. Obertop, and J. J. van Lanschot. 1999. "Prognostic value of Lauren classification and c-erbB-2 oncogene overexpression in adenocarcinoma of the esophagus and gastroesophageal junction." *Ann Surg Oncol* 6 (3):290-7.

Prenzel, N., O. M. Fischer, S. Streit, S. Hart, and A. Ullrich. 2001. "The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification." *Endocr Relat Cancer* 8 (1):11-31.

Price-Schiavi, S. A., S. Jepson, P. Li, M. Arango, P. S. Rudland, L. Yee, and K. L. Carraway. 2002. "Rat Muc4 (sialomucin complex) reduces binding of anti-ErbB2 antibodies to tumor cell surfaces, a potential mechanism for herceptin resistance." *Int J Cancer* 99 (6):783-91. doi: 10.1002/ijc.10410.

Pupa, S. M., S. Menard, D. Morelli, B. Pozzi, G. De Palo, and M. I. Colnaghi. 1993. "The extracellular domain of the c-erbB-2 oncoprotein is released from tumor cells by proteolytic cleavage." *Oncogene* 8 (11):2917-23.

Qian, X., C. M. LeVea, J. K. Freeman, W. C. Dougall, and M. I. Greene. 1994. "Heterodimerization of epidermal growth factor receptor and wild-type or kinase-deficient Neu: a mechanism of interreceptor kinase activation and transphosphorylation." *Proc Natl Acad Sci U S A* 91 (4):1500-4.

Qiu, M. Z., Q. Li, Z. Q. Wang, T. S. Liu, Q. Liu, X. L. Wei, Y. Jin, D. S. Wang, C. Ren, L. Bai, D. S. Zhang, F. H. Wang, Y. H. Li, and R. H. Xu. 2014. "HER2-positive patients receiving trastuzumab treatment have a comparable prognosis with HER2-negative advanced gastric cancer patients: a prospective cohort observation." *Int J Cancer* 134. doi: 10.1002/ijc.28559.

Queiros, P., H. Pinheiro, J. Carvalho, P. Oliveira, I. Gullo, F. Carneiro, G. M. Almeida, and C. Oliveira. 2015. "KRAS mutations in microsatellite instable gastric tumours: impact of targeted treatment and intratumoural heterogeneity." *Virchows Arch* 467 (4):383-92. doi: 10.1007/s00428-015-1823-7.

Rabinovitz, I., and A. M. Mercurio. 1997. "The integrin alpha6beta4 functions in carcinoma cell migration on laminin-1 by mediating the formation and stabilization of actin-containing motility structures." *J Cell Biol* 139 (7):1873-84.

Rexer, B. N., and C. L. Arteaga. 2013. "Optimal targeting of HER2-PI3K signaling in breast cancer: mechanistic insights and clinical implications." *Cancer Res* 73 (13):3817-20. doi: 10.1158/0008-5472.CAN-13-0687.

Ritter, C. A., M. Perez-Torres, C. Rinehart, M. Guix, T. Dugger, J. A. Engelman, and C. L. Arteaga. 2007. "Human breast cancer cells selected for resistance to trastuzumab in vivo overexpress epidermal growth factor receptor and ErbB ligands and remain dependent on the ErbB receptor network." *Clin Cancer Res* 13 (16):4909-19. doi: 10.1158/1078-0432.CCR-07-0701.

Robinson, D. R., Y. M. Wu, and S. F. Lin. 2000. "The protein tyrosine kinase family of the human genome." *Oncogene* 19 (49):5548-57. doi: 10.1038/sj.onc.1203957.

Roepstorff, K., M. V. Grandal, L. Henriksen, S. L. Knudsen, M. Lerdrup, L. Grovdal, B. M. Willumsen, and B. van Deurs. 2009. "Differential effects of EGFR ligands on endocytic sorting of the receptor." *Traffic* 10 (8):1115-27. doi: 10.1111/j.1600-0854.2009.00943.x.

Roepstorff, K., L. Grovdal, M. Grandal, M. Lerdrup, and B. van Deurs. 2008. "Endocytic downregulation of ErbB receptors: mechanisms and relevance in cancer." *Histochem Cell Biol* 129 (5):563-78. doi: 10.1007/s00418-008-0401-3.

Roosendaal, R., E. J. Kuipers, J. Buitenwerf, C. van Uffelen, S. G. Meuwissen, G. J. van Kamp, and C. M. Vandenbroucke-Grauls. 1997. "Helicobacter pylori and the birth cohort effect: evidence of a continuous decrease of infection rates in childhood." *Am J Gastroenterol* 92 (9):1480-2.

Roskoski, R., Jr. 2004. "The ErbB/HER receptor protein-tyrosine kinases and cancer." *Biochem Biophys Res Commun* 319 (1):1-11. doi: 10.1016/j.bbrc.2004.04.150.

Roskoski, R., Jr. 2014. "The ErbB/HER family of protein-tyrosine kinases and cancer." *Pharmacol Res* 79:34-74. doi: 10.1016/j.phrs.2013.11.002.

Rothlin, C. V., S. Ghosh, E. I. Zuniga, M. B. Oldstone, and G. Lemke. 2007. "TAM receptors are pleiotropic inhibitors of the innate immune response." *Cell* 131 (6):1124-36. doi: 10.1016/j.cell.2007.10.034.

Samson, P., and A. C. Lockhart. 2017. "Biologic therapy in esophageal and gastric malignancies: current therapies and future directions." *J Gastrointest Oncol* 8 (3):418-429. doi: 10.21037/jgo.2016.11.13.

Sanders, J. M., M. E. Wampole, M. L. Thakur, and E. Wickstrom. 2013. "Molecular determinants of epidermal growth factor binding: a molecular dynamics study." *PLoS One* 8 (1):e54136. doi: 10.1371/journal.pone.0054136.

Satoh, T., R. H. Xu, H. C. Chung, G. P. Sun, T. Doi, J. M. Xu, A. Tsuji, Y. Omuro, J. Li, J. W. Wang, H. Miwa, S. K. Qin, I. J. Chung, K. H. Yeh, J. F. Feng, A. Mukaiyama, M. Kobayashi, A. Ohtsu, and Y. J. Bang. 2014. "Lapatinib plus paclitaxel versus paclitaxel alone in the second-line treatment of HER2-amplified advanced gastric cancer in Asian populations: TyTAN--a randomized, phase III study." *J Clin Oncol* 32 (19):2039-49. doi: 10.1200/JCO.2013.53.6136.

Sawabu, T., H. Seno, T. Kawashima, A. Fukuda, Y. Uenoyama, M. Kawada, N. Kanda, A. Sekikawa, H. Fukui, M. Yanagita, H. Yoshibayashi, S. Satoh, Y. Sakai, T. Nakano, and T. Chiba. 2007. "Growth arrest-specific gene 6 and Axl signaling enhances gastric cancer cell survival via Akt pathway." *Mol Carcinog* 46 (2):155-64. doi: 10.1002/mc.20211.

Scaltriti, M., M. Elkabets, and J. Baselga. 2016. "Molecular Pathways: AXL, a Membrane Receptor Mediator of Resistance to Therapy." *Clin Cancer Res* 22 (6):1313-7. doi: 10.1158/1078-0432.CCR-15-1458.

Scaltriti, Maurizio, Federico Rojo, Alberto Ocana, Judit Anido, Marta Guzman, Javier Cortes, Serena Di Cosimo, Xavier Matias-Guiu, Santiago Ramon y Cajal, Joaquin Arribas, and Jose Baselga. 2007. "Expression of p95HER2, a truncated form of the HER2 receptor, and response to anti-HER2 therapies in breast cancer." *JNCI* 99 (8):628-638.

Scheuer, W., T. Friess, H. Burtscher, B. Bossenmaier, J. Endl, and M. Hasmann. 2009. "Strongly enhanced antitumor activity of trastuzumab and pertuzumab combination treatment on HER2-positive human xenograft tumor models." *Cancer Res* 69 (24):9330-6. doi: 10.1158/0008-5472.CAN-08-4597.

Schlessinger, J. 2000. "Cell signaling by receptor tyrosine kinases." *Cell* 103 (2):211-25.

Schlessinger, J., and D. Bar-Sagi. 1994. "Activation of Ras and other signaling pathways by receptor tyrosine kinases." *Cold Spring Harb Symp Quant Biol* 59:173-9.

Schneider, M. R., and E. Wolf. 2009. "The epidermal growth factor receptor ligands at a glance." *J Cell Physiol* 218 (3):460-6. doi: 10.1002/jcp.21635.

Schulte, N., M. P. Ebert, and N. Härtel. 2014. "Gastric Cancer: New Drugs - New Strategies." *Gastrointestinal Tumors* 1 (4):180-194.

Schwegler, Johann S., and Runhild Lucius. 2011. *Der Mensch - Anatomie und Physiologie.* Edited by überarb. Aufl. 5. Stuttgart: Thieme.

Segatto, O., F. Lonardo, J. H. Pierce, D. P. Bottaro, and P. P. Di Fiore. 1990. "The role of autophosphorylation in modulation of erbB-2 transforming function." *New Biol* 2 (2):187-95.

Sekiguchi, M., K. Sakakibara, and G. Fujii. 1978. "Establishment of cultured cell lines derived from a human gastric carcinoma." *Jpn J Exp Med* 48 (1):61-8.

Sereno, M., C. Aguayo, C. Guillen Ponce, C. Gomez-Raposo, F. Zambrana, M. Gomez-Lopez, and E. Casado. 2011. "Gastric tumours in hereditary cancer syndromes: clinical features, molecular biology and strategies for prevention." *Clin Transl Oncol* 13 (9):599-610.

Sergina, N. V., M. Rausch, D. Wang, J. Blair, B. Hann, K. M. Shokat, and M. M. Moasser. 2007. "Escape from HER-family tyrosine kinase inhibitor therapy by the kinase-inactive HER3." *Nature* 445 (7126):437-41. doi: 10.1038/nature05474.

Shattuck, D. L., J. K. Miller, K. L. Carraway, 3rd, and C. Sweeney. 2008. "Met receptor contributes to trastuzumab resistance of Her2-overexpressing breast cancer cells." *Cancer Res* 68 (5):1471-7. doi: 10.1158/0008-5472.CAN-07-5962.

Shi, F., S. E. Telesco, Y. Liu, R. Radhakrishnan, and M. A. Lemmon. 2010. "ErbB3/HER3 intracellular domain is competent to bind ATP and catalyze autophosphorylation." *Proc Natl Acad Sci U S A* 107 (17):7692-7. doi: 10.1073/pnas.1002753107.

Shimoyama, S. 2014. "Unraveling trastuzumab and lapatinib inefficiency in gastric cancer: Future steps (Review)." *Mol Clin Oncol* 2 (2):175-181. doi: 10.3892/mco.2013.218.

Singh, A. B., and R. C. Harris. 2005. "Autocrine, paracrine and juxtacrine signaling by EGFR ligands." *Cell Signal* 17 (10):1183-93. doi: 10.1016/j.cellsig.2005.03.026.

Sliwkowski, M. X., J. A. Lofgren, G. D. Lewis, T. E. Hotaling, B. M. Fendly, and J. A. Fox. 1999. "Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin)." *Semin Oncol* 26 (4 Suppl 12):60-70.

Smith, H. S. 1979. "In vitro properties of epithelial cell lines established from human carcinomas and nonmalignant tissue." *J Natl Cancer Inst* 62 (2):225-30.

Smith, I., M. Procter, R. D. Gelber, S. Guillaume, A. Feyereislova, M. Dowsett, A. Goldhirsch, M. Untch, G. Mariani, J. Baselga, M. Kaufmann, D. Cameron, R. Bell, J. Bergh, R. Coleman, A. Wardley, N. Harbeck, R. I. Lopez, P. Mallmann, K. Gelmon, N. Wilcken, E. Wist, P. Sanchez Rovira, M. J. Piccart-Gebhart, and Hera study team. 2007. "2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial." *Lancet* 369 (9555):29-36. doi: 10.1016/S0140-6736(07)60028-2.

Solca, F., G. Dahl, A. Zoephel, G. Bader, M. Sanderson, C. Klein, O. Kraemer, F. Himmelsbach, E. Haaksma, and G. R. Adolf. 2012. "Target binding properties and cellular activity of afatinib (BIBW 2992), an irreversible ErbB family blocker." *J Pharmacol Exp Ther* 343 (2):342-50. doi: 10.1124/jpet.112.197756.

Soltoff, S. P., and L. C. Cantley. 1996. "p120cbl is a cytosolic adapter protein that associates with phosphoinositide 3-kinase in response to epidermal growth factor in PC12 and other cells." *J Biol Chem* 271 (1):563-7.

Soltoff, S. P., K. L. Carraway, 3rd, S. A. Prigent, W. G. Gullick, and L. C. Cantley. 1994. "ErbB3 is involved in activation of phosphatidylinositol 3-kinase by epidermal growth factor." *Mol Cell Biol* 14 (6):3550-8.

Soria, J. C., E. Felip, M. Cobo, S. Lu, K. Syrigos, K. H. Lee, E. Goker, V. Georgoulias, W. Li, D. Isla, S. Z. Guclu, A. Morabito, Y. J. Min, A. Ardizzoni, S. M. Gadgeel, B. Wang, V. K. Chand, G. D. Goss, and L. UX-Lung 8 Investigators. 2015. "Afatinib versus erlotinib as second-line treatment of patients with advanced squamous cell carcinoma of the lung (LUX-Lung 8): an open-label randomised controlled phase 3 trial." *Lancet Oncol* 16 (8):897-907. doi: 10.1016/S1470-2045(15)00006-6.

Spector, N. L., W. Xia, H. Burris, 3rd, H. Hurwitz, E. C. Dees, A. Dowlati, B. O'Neil, B. Overmoyer, P. K. Marcom, K. L. Blackwell, D. A. Smith, K. M. Koch, A. Stead, S. Mangum, M. J. Ellis, L. Liu, A. K. Man, T. M. Bremer, J. Harris, and S. Bacus. 2005. "Study of the biologic effects of lapatinib, a reversible inhibitor of ErbB1 and ErbB2 tyrosine kinases, on tumor growth and survival pathways in patients with advanced malignancies." *J Clin Oncol* 23 (11):2502-12. doi: 10.1200/JCO.2005.12.157.

Stommel, J. M., A. C. Kimmelman, H. Ying, R. Nabioullin, A. H. Ponugoti, R. Wiedemeyer, A. H. Stegh, J. E. Bradner, K. L. Ligon, C. Brennan, L. Chin, and R. A. DePinho. 2007. "Coactivation of receptor tyrosine kinases affects the response of tumor cells to targeted therapies." *Science* 318 (5848):287-90. doi: 10.1126/science.1142946.

Swain, S. M., J. Baselga, S. B. Kim, J. Ro, V. Semiglazov, M. Campone, E. Ciruelos, J. M. Ferrero, A. Schneeweiss, S. Heeson, E. Clark, G. Ross, M. C. Benyunes, J. Cortes, and Cleopatra Study Group. 2015. "Pertuzumab, trastuzumab, and docetaxel in HER2-positive metastatic breast cancer." *N Engl J Med* 372 (8):724-34. doi: 10.1056/NEJMoa1413513.

Takeda, S., T. Tanigawa, T. Watanabe, H. Tatsuwaki, Y. Nadatani, K. Otani, Y. Nagami, F. Tanaka, N. Kamata, H. Yamagami, M. Shiba, K. Tominaga, Y. Fujiwara, K. Muguruma, M. Ohira, K. Hirakawa, and T. Arakawa. 2016. "Reduction of prostaglandin transporter predicts poor prognosis associated with angiogenesis in gastric adenocarcinoma." *J Gastroenterol Hepatol* 31 (2):376-83. doi: 10.1111/jgh.13079.

Takeuchi, K., S. Shibamoto, M. Hayakawa, T. Hori, K. Miyazawa, N. Kitamura, and F. Ito. 1996. "Hepatocyte growth factor (HGF)-induced cell migration is negatively

modulated by epidermal growth factor through tyrosine phosphorylation of the HGF receptor." *Exp Cell Res* 223 (2):420-5. doi: 10.1006/excr.1996.0097.

Takikita, M., R. Xie, J. Y. Chung, H. Cho, K. Ylaya, S. M. Hong, C. A. Moskaluk, and S. M. Hewitt. 2011. "Membranous expression of Her3 is associated with a decreased survival in head and neck squamous cell carcinoma." *J Transl Med* 9:126. doi: 10.1186/1479-5876-9-126.

Tanizaki, J., I. Okamoto, K. Takezawa, S. Tsukioka, J. Uchida, M. Kiniwa, M. Fukuoka, and K. Nakagawa. 2010. "Synergistic antitumor effect of S-1 and HER2-targeting agents in gastric cancer with HER2 amplification." *Mol Cancer Ther* 9 (5):1198-207. doi: 10.1158/1535-7163.MCT-10-0045.

Tanner, M., M. Hollmen, T. T. Junttila, A. I. Kapanen, S. Tommola, Y. Soini, H. Helin, J. Salo, H. Joensuu, E. Sihvo, K. Elenius, and J. Isola. 2005. "Amplification of HER-2 in gastric carcinoma: association with Topoisomerase IIalpha gene amplification, intestinal type, poor prognosis and sensitivity to trastuzumab." *Ann Oncol* 16 (2):273-8. doi: 10.1093/annonc/mdi064.

Tao, R. H., and I. N. Maruyama. 2008. "All EGF(ErbB) receptors have preformed homo- and heterodimeric structures in living cells." *J Cell Sci* 121 (Pt 19):3207-17. doi: 10.1242/jcs.033399.

Thorlacius, S., G. Olafsdottir, L. Tryggvadottir, S. Neuhausen, J. G. Jonasson, S. V. Tavtigian, H. Tulinius, H. M. Ogmundsdottir, and J. E. Eyfjord. 1996. "A single BRCA2 mutation in male and female breast cancer families from Iceland with varied cancer phenotypes." *Nat Genet* 13 (1):117-9. doi: 10.1038/ng0596-117.

Tkachenko, M. A., N. Z. Zhannat, L. V. Erman, E. L. Blashenkova, S. V. Isachenko, O. B. Isachenko, D. Y. Graham, and H. M. Malaty. 2007. "Dramatic changes in the prevalence of Helicobacter pylori infection during childhood: a 10-year follow-up study in Russia." *J Pediatr Gastroenterol Nutr* 45 (4):428-32. doi: 10.1097/MPG.0b013e318064589f.

Tomioka, H., T. Mukohara, Y. Kataoka, R. C. Ekyalongo, Y. Funakoshi, Y. Imai, N. Kiyota, Y. Fujiwara, and H. Minami. 2012. "Inhibition of the mTOR/S6K signal is necessary to enhance fluorouracil-induced apoptosis in gastric cancer cells with HER2 amplification." *Int J Oncol* 41 (2):551-8. doi: 10.3892/ijo.2012.1485.

Tse, C., A. S. Gauchez, W. Jacot, and P. J. Lamy. 2012. "HER2 shedding and serum HER2 extracellular domain: biology and clinical utility in breast cancer." *Cancer Treat Rev* 38 (2):133-42. doi: 10.1016/j.ctrv.2011.03.008.

Turke, A. B., K. Zejnullahu, Y. L. Wu, Y. Song, D. Dias-Santagata, E. Lifshits, L. Toschi, A. Rogers, T. Mok, L. Sequist, N. I. Lindeman, C. Murphy, S. Akhavanfard, B. Y. Yeap, Y. Xiao, M. Capelletti, A. J. Iafrate, C. Lee, J. G. Christensen, J. A. Engelman, and P. A. Janne. 2010. "Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC." *Cancer Cell* 17 (1):77-88. doi: 10.1016/j.ccr.2009.11.022.

Tym, J. E., C. Mitsopoulos, E. A. Coker, P. Razaz, A. C. Schierz, A. A. Antolin, and B. Al-Lazikani. 2016. "canSAR: an updated cancer research and drug discovery knowledgebase." *Nucleic Acids Res* 44 (D1):D938-43. doi: 10.1093/nar/gkv1030.

Tzahar, E., H. Waterman, X. Chen, G. Levkowitz, D. Karunagaran, S. Lavi, B. J. Ratzkin, and Y. Yarden. 1996. "A hierarchical network of interreceptor interactions

determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor." *Mol Cell Biol* 16 (10):5276-87.

Uribe, D. J., E. K. Mandell, A. Watson, J. D. Martinez, J. A. Leighton, S. Ghosh, and C. V. Rothlin. 2017. "The receptor tyrosine kinase AXL promotes migration and invasion in colorectal cancer." *PLoS One* 12 (7):e0179979. doi: 10.1371/journal.pone.0179979.

Ushijima, T., and M. Sasako. 2004. "Focus on gastric cancer." *Cancer Cell* 5 (2):121-5.

Valabrega, G., F. Montemurro, and M. Aglietta. 2007. "Trastuzumab: mechanism of action, resistance and future perspectives in HER2-overexpressing breast cancer." *Ann Oncol* 18 (6):977-84. doi: 10.1093/annonc/mdl475.

Van Cutsem, E., C. H. Kohne, I. Lang, G. Folprecht, M. P. Nowacki, S. Cascinu, I. Shchepotin, J. Maurel, D. Cunningham, S. Tejpar, M. Schlichting, A. Zubel, I. Celik, P. Rougier, and F. Ciardiello. 2011. "Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status." *J Clin Oncol* 29 (15):2011-9. doi: 10.1200/JCO.2010.33.5091.

Van Cutsem, E., V. M. Moiseyenko, S. Tjulandin, A. Majlis, M. Constenla, C. Boni, A. Rodrigues, M. Fodor, Y. Chao, E. Voznyi, M. L. Risse, J. A. Ajani, and V. Study Group. 2006. "Phase III study of docetaxel and cisplatin plus fluorouracil compared with cisplatin and fluorouracil as first-line therapy for advanced gastric cancer: a report of the V325 Study Group." *J Clin Oncol* 24 (31):4991-7. doi: 10.1200/JCO.2006.06.8429.

Vanhoefer, U., P. Rougier, H. Wilke, M. P. Ducreux, A. J. Lacave, E. Van Cutsem, M. Planker, J. G. Santos, P. Piedbois, B. Paillot, H. Bodenstein, H. J. Schmoll, H. Bleiberg, B. Nordlinger, M. L. Couvreur, B. Baron, and J. A. Wils. 2000. "Final results of a randomized phase III trial of sequential high-dose methotrexate, fluorouracil, and doxorubicin versus etoposide, leucovorin, and fluorouracil versus infusional fluorouracil and cisplatin in advanced gastric cancer: A trial of the European Organization for Research and Treatment of Cancer Gastrointestinal Tract Cancer Cooperative Group." *J Clin Oncol* 18 (14):2648-57. doi: 10.1200/JCO.2000.18.14.2648.

Varley, J. M., G. McGown, M. Thorncroft, K. J. Tricker, M. D. Teare, M. F. Santibanez-Koref, J. Martin, J. M. Birch, and D. G. Evans. 1995. "An extended Li-Fraumeni kindred with gastric carcinoma and a codon 175 mutation in TP53." *J Med Genet* 32 (12):942-5.

Varnum, B. C., C. Young, G. Elliott, A. Garcia, T. D. Bartley, Y. W. Fridell, R. W. Hunt, G. Trail, C. Clogston, R. J. Toso, and et al. 1995. "Axl receptor tyrosine kinase stimulated by the vitamin K-dependent protein encoded by growth-arrest-specific gene 6." *Nature* 373 (6515):623-6. doi: 10.1038/373623a0.

Velho, S., C. Oliveira, A. Ferreira, A. C. Ferreira, G. Suriano, S. Schwartz, Jr., A. Duval, F. Carneiro, J. C. Machado, R. Hamelin, and R. Seruca. 2005. "The prevalence of PIK3CA mutations in gastric and colon cancer." *Eur J Cancer* 41 (11):1649-54. doi: 10.1016/j.ejca.2005.04.022.

Vermorken, J. B., R. Mesia, F. Rivera, E. Remenar, A. Kawecki, S. Rottey, J. Erfan, D. Zabolotnyy, H. R. Kienzer, D. Cupissol, F. Peyrade, M. Benasso, I. Vynnychenko, D. De Raucourt, C. Bokemeyer, A. Schueler, N. Amellal, and R. Hitt. 2008.

"Platinum-based chemotherapy plus cetuximab in head and neck cancer." *N Engl J Med* 359 (11):1116-27. doi: 10.1056/NEJMoa0802656.
Vincenzi, B., A. Zoccoli, F. Pantano, O. Venditti, and S. Galluzzo. 2010. "Cetuximab: from bench to bedside." *Curr Cancer Drug Targets* 10 (1):80-95.
Vogelstein, B., and K. W. Kinzler. 1993. "The multistep nature of cancer." *Trends Genet* 9 (4):138-41.
Vogiatzi, P., C. Vindigni, F. Roviello, A. Renieri, and A. Giordano. 2007. "Deciphering the underlying genetic and epigenetic events leading to gastric carcinogenesis." *J Cell Physiol* 211 (2):287-95. doi: 10.1002/jcp.20982.
Vouri, M., D. R. Croucher, S. P. Kennedy, Q. An, G. J. Pilkington, and S. Hafizi. 2016. "Axl-EGFR receptor tyrosine kinase hetero-interaction provides EGFR with access to pro-invasive signalling in cancer cells." *Oncogenesis* 5 (10):e266. doi: 10.1038/oncsis.2016.66.
Vu, T., M. X. Sliwkowski, and F. X. Claret. 2014. "Personalized drug combinations to overcome trastuzumab resistance in HER2-positive breast cancer." *Biochim Biophys Acta* 1846 (2):353-65. doi: 10.1016/j.bbcan.2014.07.007.
Waddell, T., I. Chau, D. Cunningham, D. Gonzalez, A. F. Okines, C. Okines, A. Wotherspoon, C. Saffery, G. Middleton, J. Wadsley, D. Ferry, W. Mansoor, T. Crosby, F. Coxon, D. Smith, J. Waters, T. Iveson, S. Falk, S. Slater, C. Peckitt, and Y. Barbachano. 2013. "Epirubicin, oxaliplatin, and capecitabine with or without panitumumab for patients with previously untreated advanced oesophagogastric cancer (REAL3): a randomised, open-label phase 3 trial." *Lancet Oncol* 14 (6):481-9. doi: 10.1016/S1470-2045(13)70096-2.
Wadhwa, R., S. Song, J. S. Lee, Y. Yao, Q. Wei, and J. A. Ajani. 2013. "Gastric cancer-molecular and clinical dimensions." *Nat Rev Clin Oncol* 10 (11):643-55. doi: 10.1038/nrclinonc.2013.170.
Wagner, A. D., W. Grothe, J. Haerting, G. Kleber, A. Grothey, and W. E. Fleig. 2006. "Chemotherapy in advanced gastric cancer: a systematic review and meta-analysis based on aggregate data." *J Clin Oncol* 24 (18):2903-9. doi: 10.1200/JCO.2005.05.0245.
Wainberg, Z. A., A. Anghel, A. J. Desai, R. Ayala, T. Luo, B. Safran, M. S. Fejzo, J. R. Hecht, D. J. Slamon, and R. S. Finn. 2010. "Lapatinib, a dual EGFR and HER2 kinase inhibitor, selectively inhibits HER2-amplified human gastric cancer cells and is synergistic with trastuzumab in vitro and in vivo." *Clin Cancer Res* 16 (5):1509-19. doi: 10.1158/1078-0432.CCR-09-1112.
Wang, Q., X. Zhang, E. Shen, J. Gao, F. Cao, X. Wang, Y. Li, T. Tian, J. Wang, Z. Chen, J. Wang, and L. Shen. 2016. "The anti-HER3 antibody in combination with trastuzumab exerts synergistic antitumor activity in HER2-positive gastric cancer." *Cancer Lett* 380 (1):20-30. doi: 10.1016/j.canlet.2016.06.005.
Wang, S. E., B. Xiang, M. Guix, M. G. Olivares, J. Parker, C. H. Chung, A. Pandiella, and C. L. Arteaga. 2008. "Transforming growth factor beta engages TACE and ErbB3 to activate phosphatidylinositol-3 kinase/Akt in ErbB2-overexpressing breast cancer and desensitizes cells to trastuzumab." *Mol Cell Biol* 28 (18):5605-20. doi: 10.1128/MCB.00787-08.

Wang, X. Q., P. D. Terry, and H. Yan. 2009. "Review of salt consumption and stomach cancer risk: epidemiological and biological evidence." *World J Gastroenterol* 15 (18):2204-13.
Warren, J. R., and B. Marshall. 1983. "Unidentified curved bacilli on gastric epithelium in active chronic gastritis." *Lancet* 1 (8336):1273-5.
Weaver, V. M., S. Lelievre, J. N. Lakins, M. A. Chrenek, J. C. Jones, F. Giancotti, Z. Werb, and M. J. Bissell. 2002. "beta4 integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium." *Cancer Cell* 2 (3):205-16.
Weinberg, R. A. 2014. *The Biology of Cancer*. Vol. 2nd: Garland Science.
Wells, A. 1999. "EGF receptor." *Int J Biochem Cell Biol* 31 (6):637-43.
Wells, A. 2000. "Tumor invasion: role of growth factor-induced cell motility." *Adv Cancer Res* 78:31-101.
Wells, A., J. Grahovac, S. Wheeler, B. Ma, and D. Lauffenburger. 2013. "Targeting tumor cell motility as a strategy against invasion and metastasis." *Trends Pharmacol Sci* 34 (5):283-9. doi: 10.1016/j.tips.2013.03.001.
Wells, A., J. Kassis, J. Solava, T. Turner, and D. A. Lauffenburger. 2002. "Growth factor-induced cell motility in tumor invasion." *Acta Oncol* 41 (2):124-30.
Wheeler, D. L., S. Huang, T. J. Kruser, M. M. Nechrebecki, E. A. Armstrong, S. Benavente, V. Gondi, K. T. Hsu, and P. M. Harari. 2008. "Mechanisms of acquired resistance to cetuximab: role of HER (ErbB) family members." *Oncogene* 27 (28):3944-56. doi: 10.1038/onc.2008.19.
Wind, S., D. Schnell, T. Ebner, M. Freiwald, and P. Stopfer. 2016. "Clinical Pharmacokinetics and Pharmacodynamics of Afatinib." *Clin Pharmacokinet*. doi: 10.1007/s40262-016-0440-1.
Wittekind, C. 2015. "The development of the TNM classification of gastric cancer." *Pathol Int* 65 (8):399-403. doi: 10.1111/pin.12306.
Wolf-Yadlin, A., N. Kumar, Y. Zhang, S. Hautaniemi, M. Zaman, H. D. Kim, V. Grantcharova, D. A. Lauffenburger, and F. M. White. 2006. "Effects of HER2 overexpression on cell signaling networks governing proliferation and migration." *Mol Syst Biol* 2:54. doi: 10.1038/msb4100094.
Wolf, K., Y. I. Wu, Y. Liu, J. Geiger, E. Tam, C. Overall, M. S. Stack, and P. Friedl. 2007. "Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion." *Nat Cell Biol* 9 (8):893-904. doi: 10.1038/ncb1616.
Woo, J., S. A. Cohen, and J. E. Grim. 2015. "Targeted therapy in gastroesophageal cancers: past, present and future." *Gastroenterol Rep (Oxf)* 3 (4):316-29. doi: 10.1093/gastro/gov052.
Wroblewski, L. E., R. M. Peek, Jr., and K. T. Wilson. 2010. "Helicobacter pylori and gastric cancer: factors that modulate disease risk." *Clin Microbiol Rev* 23 (4):713-39. doi: 10.1128/CMR.00011-10.
Yaghoobi, M., R. Bijarchi, and S. A. Narod. 2010. "Family history and the risk of gastric cancer." *Br J Cancer* 102 (2):237-42. doi: 10.1038/sj.bjc.6605380.
Yakes, F. M., W. Chinratanalab, C. A. Ritter, W. King, S. Seelig, and C. L. Arteaga. 2002. "Herceptin-induced inhibition of phosphatidylinositol-3 kinase and Akt Is required for antibody-mediated effects on p27, cyclin D1, and antitumor action." *Cancer Res* 62 (14):4132-41.

Yamashita-Kashima, Y., S. Iijima, K. Yorozu, K. Furugaki, M. Kurasawa, M. Ohta, and K. Fujimoto-Ouchi. 2011. "Pertuzumab in combination with trastuzumab shows significantly enhanced antitumor activity in HER2-positive human gastric cancer xenograft models." *Clin Cancer Res* 17 (15):5060-70. doi: 10.1158/1078-0432.CCR-10-2927.

Yamauchi, H., A. O'Neill, R. Gelman, W. Carney, D. Y. Tenney, S. Hosch, and D. F. Hayes. 1997. "Prediction of response to antiestrogen therapy in advanced breast cancer patients by pretreatment circulating levels of extracellular domain of the HER-2/c-neu protein." *J Clin Oncol* 15 (7):2518-25.

Yanagihara, K., A. Ito, T. Toge, and M. Numoto. 1993. "Antiproliferative effects of isoflavones on human cancer cell lines established from the gastrointestinal tract." *Cancer Res* 53 (23):5815-21.

Yanagihara, K., T. Seyama, M. Tsumuraya, N. Kamada, and K. Yokoro. 1991. "Establishment and characterization of human signet ring cell gastric carcinoma cell lines with amplification of the c-myc oncogene." *Cancer Res* 51 (1):381-6.

Yang, W., A. Raufi, and S. J. Klempner. 2014. "Targeted therapy for gastric cancer: molecular pathways and ongoing investigations." *Biochim Biophys Acta* 1846 (1):232-7. doi: 10.1016/j.bbcan.2014.05.003.

Yarden, Y. 2001a. "Biology of HER2 and its importance in breast cancer." *Oncology* 61 Suppl 2:1-13. doi: 55396.

Yarden, Y. 2001b. "The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities." *Eur J Cancer* 37 Suppl 4:S3-8.

Yarden, Y., and G. Pines. 2012. "The ERBB network: at last, cancer therapy meets systems biology." *Nat Rev Cancer* 12 (8):553-63. doi: 10.1038/nrc3309.

Yarden, Y., and M. X. Sliwkowski. 2001. "Untangling the ErbB signalling network." *Nat Rev Mol Cell Biol* 2 (2):127-37. doi: 10.1038/35052073.

Yewale, C., D. Baradia, I. Vhora, S. Patil, and A. Misra. 2013. "Epidermal growth factor receptor targeting in cancer: a review of trends and strategies." *Biomaterials* 34 (34):8690-707. doi: 10.1016/j.biomaterials.2013.07.100.

Yokoyama, H., Y. Ikehara, Y. Kodera, S. Ikehara, Y. Yatabe, Y. Mochizuki, M. Koike, M. Fujiwara, A. Nakao, M. Tatematsu, and H. Nakanishi. 2006. "Molecular basis for sensitivity and acquired resistance to gefitinib in HER2-overexpressing human gastric cancer cell lines derived from liver metastasis." *Br J Cancer* 95 (11):1504-13. doi: 10.1038/sj.bjc.6603459.

Yokozaki, H. 2000. "Molecular characteristics of eight gastric cancer cell lines established in Japan." *Pathol Int* 50 (10):767-77.

Yonsei, University. 2017. Afatinib, paclitaxel, 2nd Line, advanced gastric cancer. https://ClinicalTrials.gov/show/NCT02501603.

Yotsumoto, F., E. Oki, E. Tokunaga, Y. Maehara, M. Kuroki, and S. Miyamoto. 2010. "HB-EGF orchestrates the complex signals involved in triple-negative and trastuzumab-resistant breast cancer." *Int J Cancer* 127 (11):2707-17. doi: 10.1002/ijc.25472.

Yuan, H. H., Y. N. Yang, J. H. Zhou, Y. J. Li, L. Y. Wang, J. W. Qin, T. Liu, Z. Z. Li, Q. X. Zhou, X. L. Wei, T. T. Zhang, P. Huang, W. J. Zhang, L. Liu, X. X. Du, and Y. Han. 2017. "siRNA-mediated inactivation of HER3 improves the antitumour activity and sensitivity of gefitinib in gastric cancer cells." *Oncotarget* 8 (32):52584-52593. doi: 10.18632/oncotarget.17526.

Yun, C., L. Gang, G. Rongmin, W. Xu, M. Xuezhi, and C. Huanqiu. 2015. "Essential role of Her3 in two signaling transduction patterns: Her2/Her3 and MET/Her3 in proliferation of human gastric cancer." *Mol Carcinog* 54 (12):1700-9. doi: 10.1002/mc.22241.

Zabrecky, J. R., T. Lam, S. J. McKenzie, and W. Carney. 1991. "The extracellular domain of p185/neu is released from the surface of human breast carcinoma cells, SK-BR-3." *J Biol Chem* 266 (3):1716-20.

Zali, H., M. Rezaei-Tavirani, and M. Azodi. 2011. "Gastric cancer: prevention, risk factors and treatment." *Gastroenterol Hepatol Bed Bench* 4 (4):175-85.

Zandi, R., A. B. Larsen, P. Andersen, M. T. Stockhausen, and H. S. Poulsen. 2007. "Mechanisms for oncogenic activation of the epidermal growth factor receptor." *Cell Signal* 19 (10):2013-23. doi: 10.1016/j.cellsig.2007.06.023.

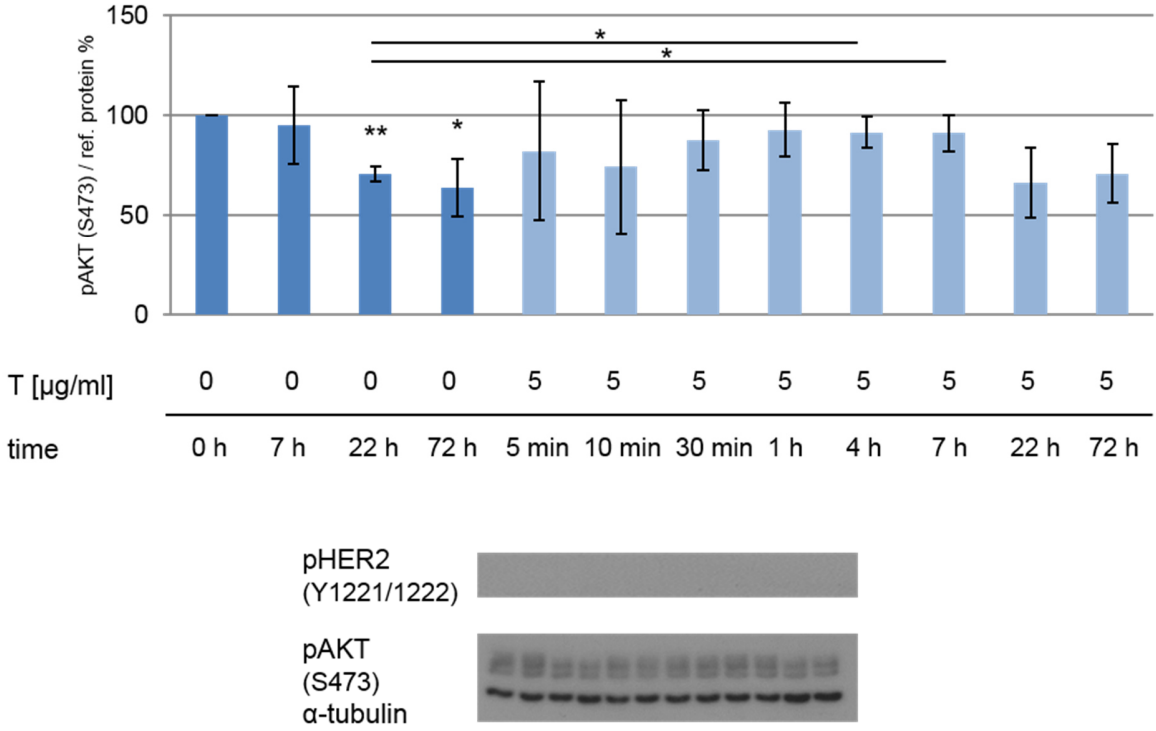
Zanghieri, G., C. Di Gregorio, C. Sacchetti, R. Fante, R. Sassatelli, G. Cannizzo, A. Carriero, and M. Ponz de Leon. 1990. "Familial occurrence of gastric cancer in the 2-year experience of a population-based registry." *Cancer* 66 (9):2047-51.

Zhang, Y. X., P. G. Knyazev, Y. V. Cheburkin, K. Sharma, Y. P. Knyazev, L. Orfi, I. Szabadkai, H. Daub, G. Keri, and A. Ullrich. 2008. "AXL is a potential target for therapeutic intervention in breast cancer progression." *Cancer Res* 68 (6):1905-15. doi: 10.1158/0008-5472.CAN-07-2661.

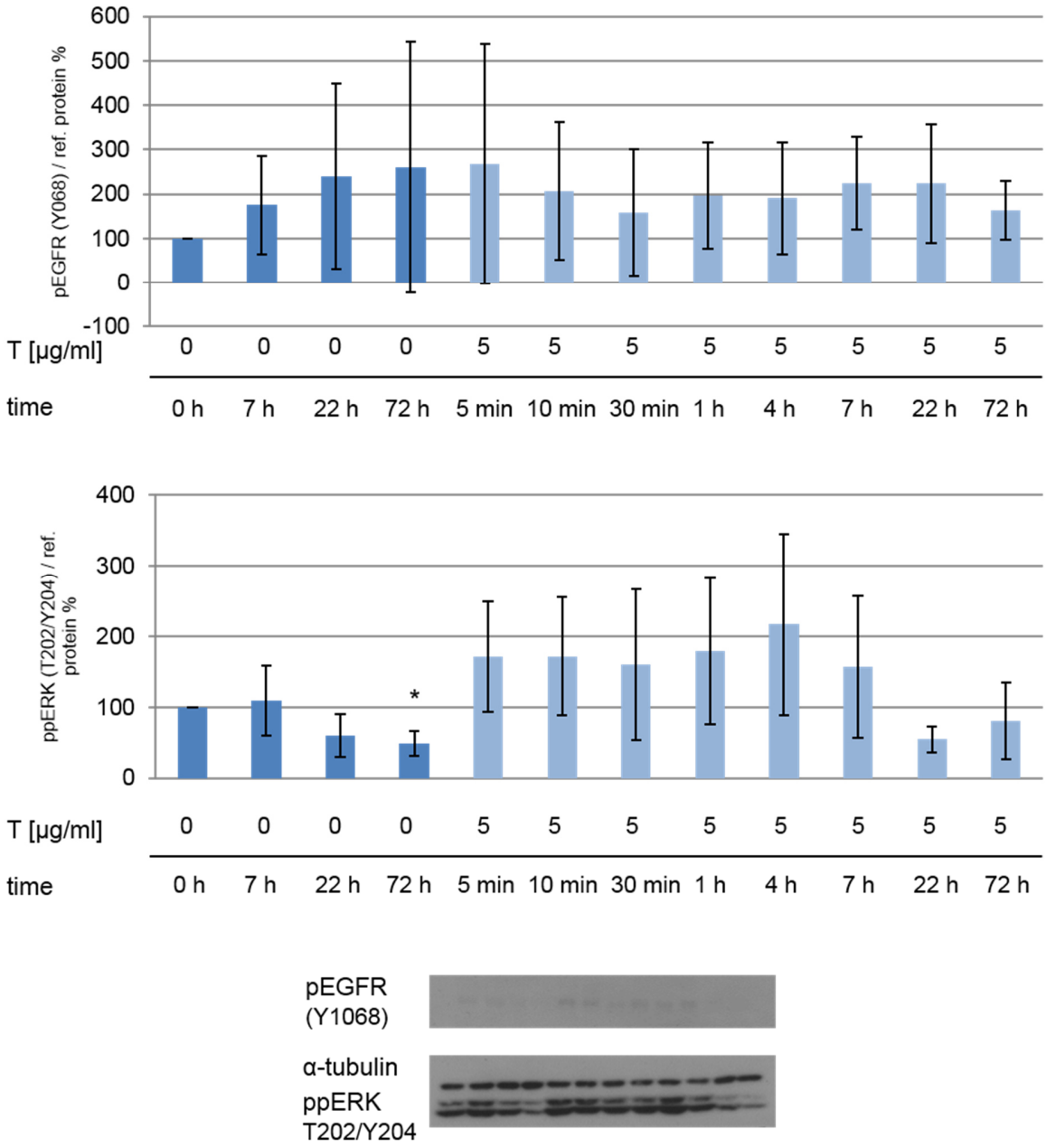
Zheng, L., W. Tan, J. Zhang, D. Yuan, J. Yang, and H. Liu. 2014. "Combining trastuzumab and cetuximab combats trastuzumab-resistant gastric cancer by effective inhibition of EGFR/ErbB2 heterodimerization and signaling." *Cancer Immunol Immunother* 63 (6):581-6. doi: 10.1007/s00262-014-1541-z.

Zutter, M. M. 2007. "Integrin-mediated adhesion: tipping the balance between chemosensitivity and chemoresistance." *Adv Exp Med Biol* 608:87-100.

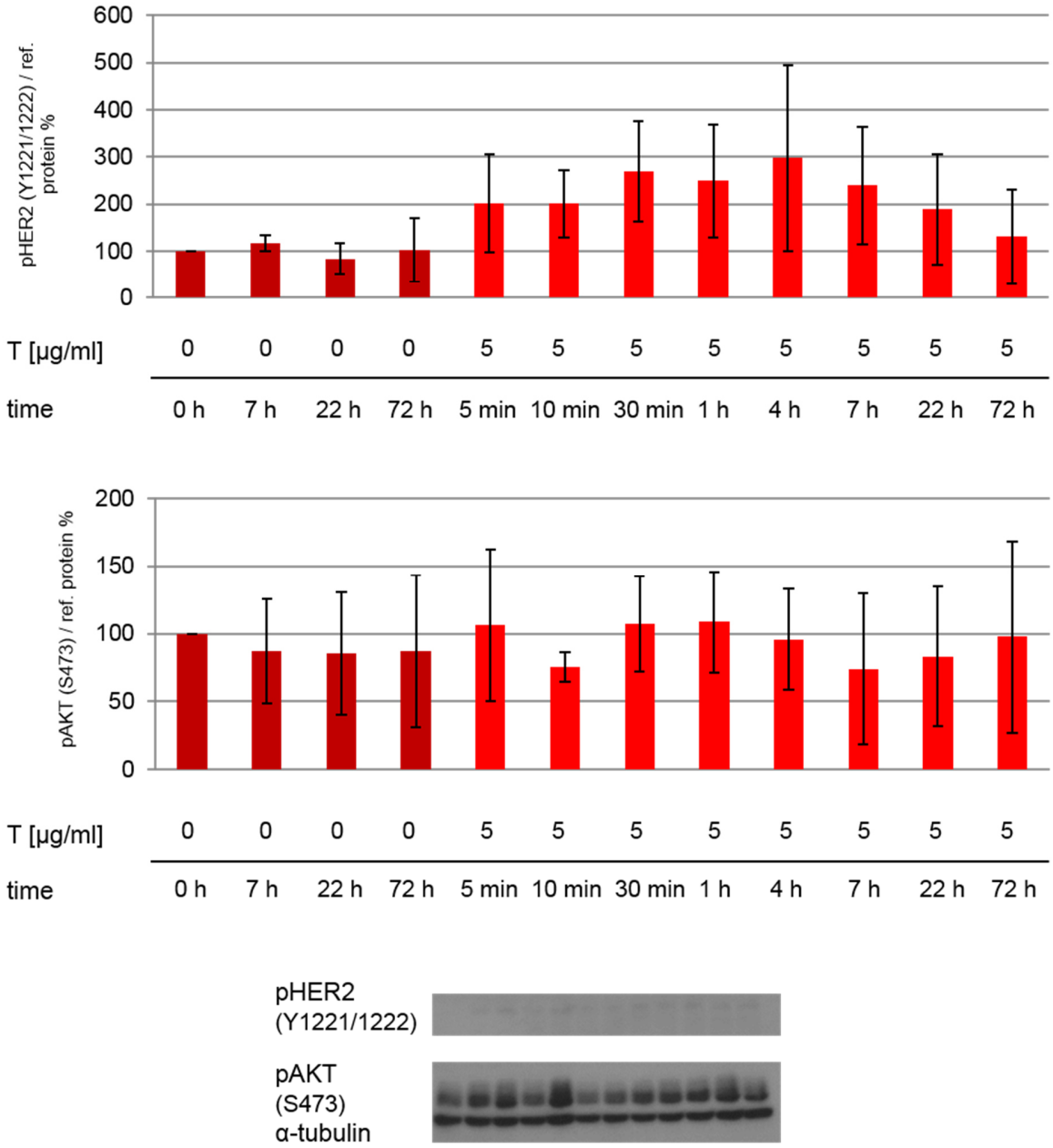
7 APPENDIX



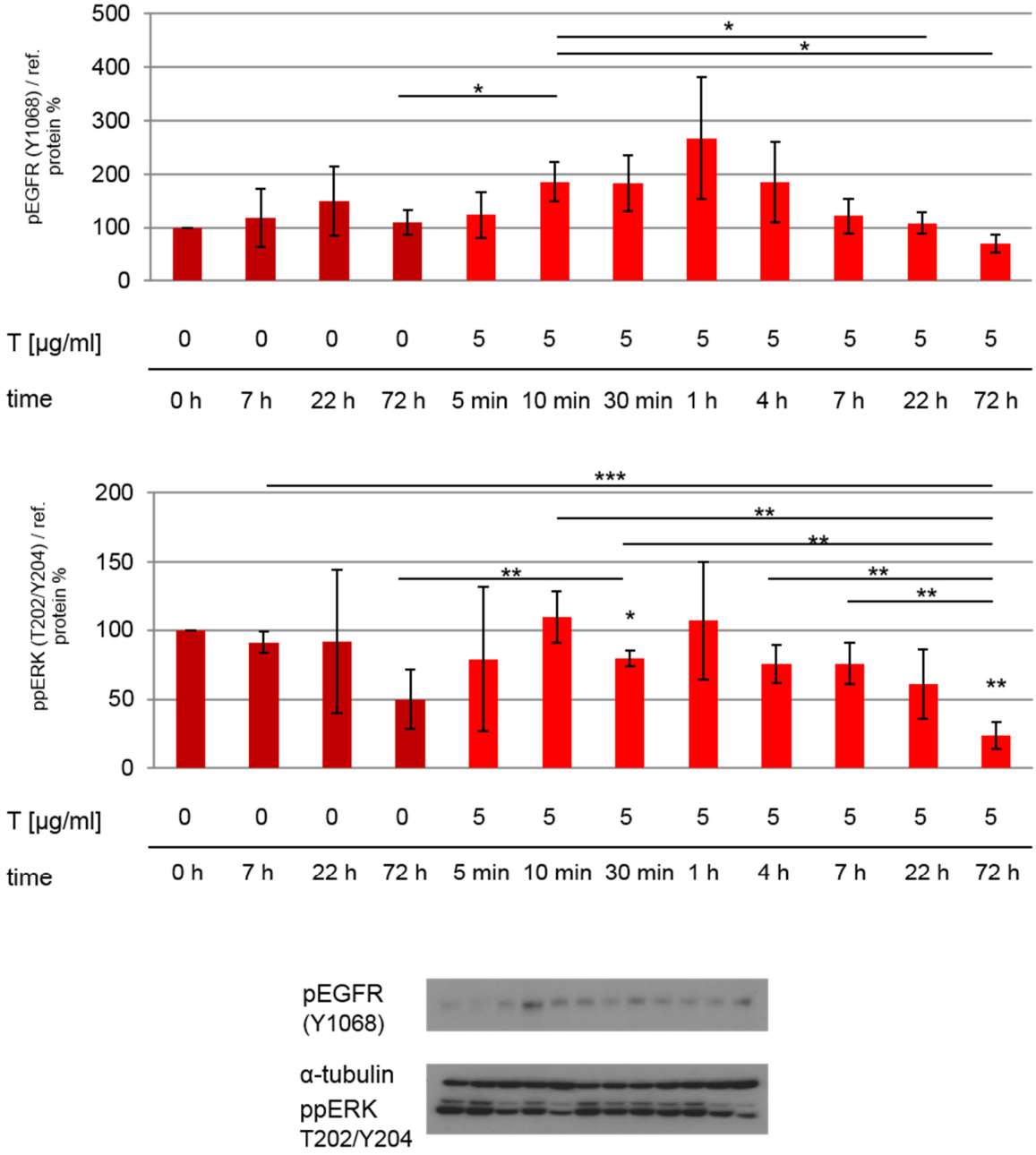
Suppl. Figure: 1: Kinetic effects of trastuzumab for pHER2 and pAKT in MKN1.
In order to analyze time dependent effects of trastuzumab on the activation of HER2 and AKT in MKN1 a kinetic was prepared. Total lysates of untreated cells and cells treated with 5 µg/ml trastuzumab for different time points were analyzed. Untreated samples were prepared at 0, 7, 22 and 72 hours to visualize the effect of the culture time itself. Therefore, the untreated samples were set to 100 % to visualize the effect of the treatment and the culture time. For pAKT significant changes were detectable. Hardly any signals were detectable for pHER2, therefore no reliable analysis was possible for pHER2. The mean values of three independent experiments are shown with one picture as an exemplary result. Significant effects are indicated by * $0.01 < \text{p-value} \leq 0.05$ or ** $0.001 < \text{p-value} \leq 0.01$ (one-sample and two-sided Welch's t-test).



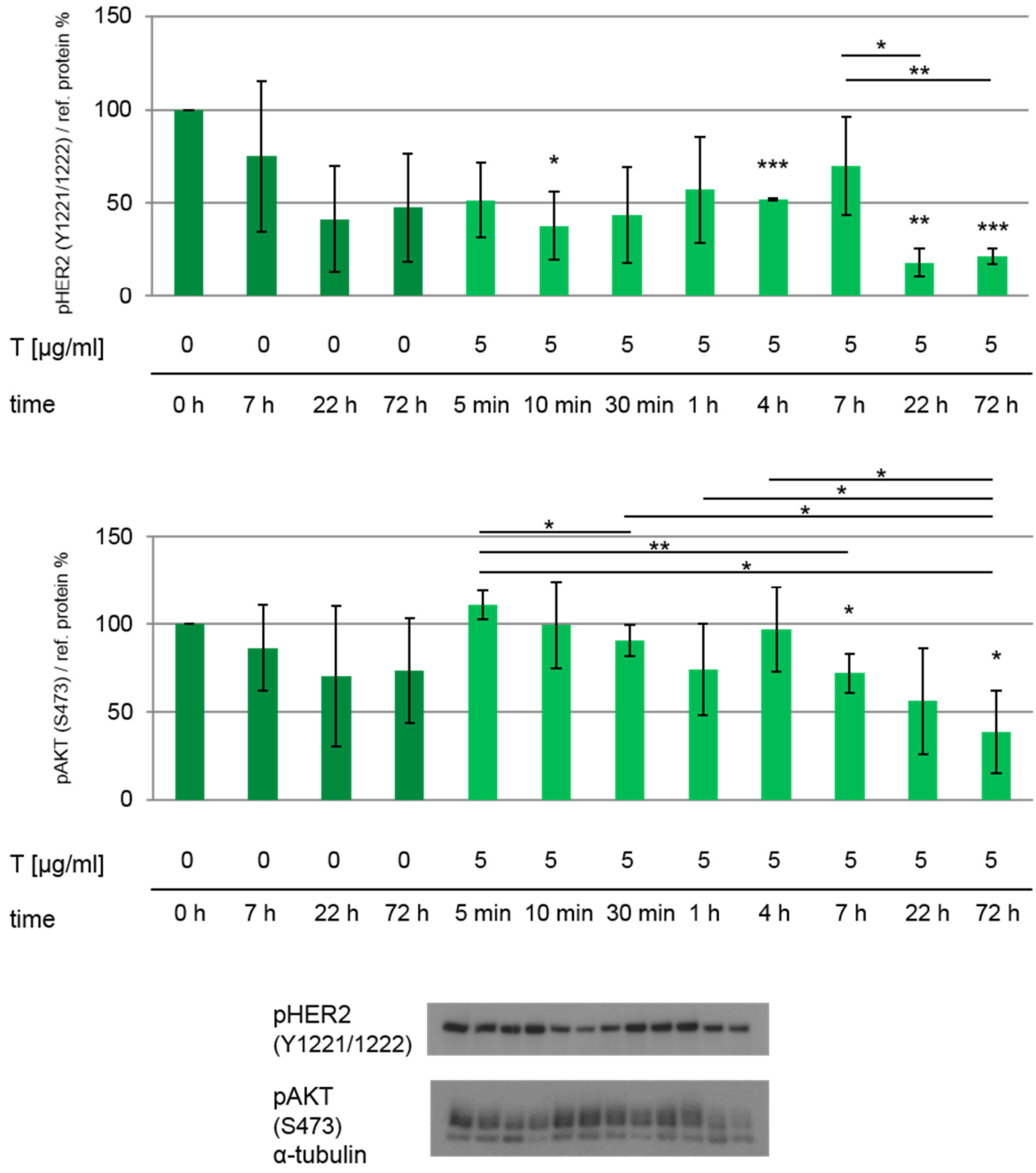
Suppl. Figure: 2: Kinetic effects of trastuzumab for pEGFR and pERK in MKN1.
In order to analyze time dependent effects of trastuzumab on the activation of EGFR and ERK in MKN1 a kinetic was prepared. Total lysates of untreated cells and cells treated with 5 µg/ml trastuzumab for different time points were analyzed. Untreated samples were prepared at 0, 7, 22 and 72 hours to visualize the effect of the culture time itself. Therefore, the untreated samples were set to 100 % to visualize the effect of the treatment and the culture time. For pERK a culture time depending effect was detectable whereas pEGFR was hardly measurable. The mean values of three independent experiments are shown with one picture as an exemplary result. Significant effects are indicated by * 0.01 < p-value ≤ 0.05 (one-sample and two-sided Welch's t-test).



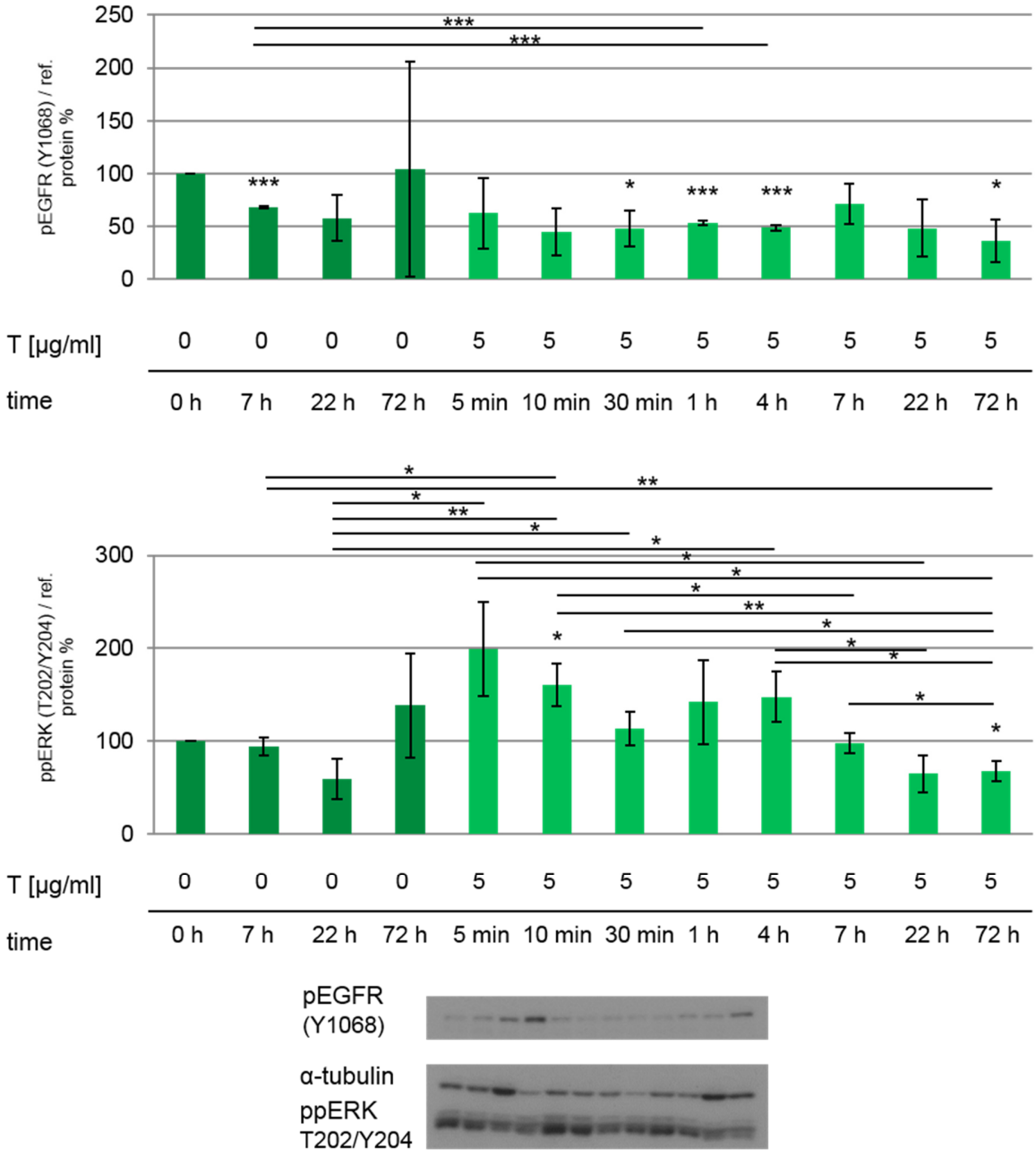
Suppl. Figure: 3: Kinetic effects of trastuzumab for pHER2 and pAKT in MKN7.
In order to analyze time dependent effects of trastuzumab on the activation of HER2 and AKT in MKN7 a kinetic was prepared. Total lysates of untreated cells and cells treated with 5 µg/ml trastuzumab for different time points were analyzed. Untreated samples were prepared at 0, 7, 22 and 72 hours to visualize the effect of the culture time itself. Therefore, the untreated samples were set to 100 % to visualize the effect of the treatment and the culture time. No changes were detected for pAKT and pHER2. The signals for pHER2 were slight. The mean values of three independent experiments are shown with one picture as an exemplary result.



Suppl. Figure: 4: Kinetic effects of trastuzumab for pEGFR and pERK in MKN7.
In order to analyze time dependent effects of trastuzumab on the activation of EGFR and ERK in MKN7 a kinetic was prepared. Total lysates of untreated cells and cells treated with 5 µg/ml trastuzumab for different time points were analyzed. Untreated samples were prepared at 0, 7, 22 and 72 hours to visualize the effect of the culture time itself. Therefore, the untreated samples were set to 100 % to visualize the effect of the treatment and the culture time. Both proteins were significantly influenced by trastuzumab. The mean values of three independent experiments are shown with one picture as an exemplary result. Significant effects are indicated by * 0.01 < p-value ≤ 0.05, ** 0.001 < p-value ≤ 0.01 or ***< 0.001 (one-sample and two-sided Welch's t-test).



Suppl. Figure: 5: Kinetic effects of trastuzumab for pHER2 and pAKT in NCI-N87.
In order to analyze time dependent effects of trastuzumab on the activation of HER2 and AKT in NCI-N87 a kinetic was prepared. Total lysates of untreated cells and cells treated with 5 µg/ml trastuzumab for different time points were analyzed. Untreated samples were prepared at 0, 7, 22 and 72 hours to visualize the effect of the culture time itself. Therefore, the untreated samples were set to 100 % to visualize the effect of the treatment and the culture time. Both proteins were significantly influenced by trastuzumab. The mean values of three independent experiments are shown with one picture as an exemplary result. Significant effects are indicated by * 0.01 < p-value ≤ 0.05, ** 0.001 < p-value ≤ 0.01 or ***< 0.001 (one-sample and two-sided Welch's t-test).



Suppl. Figure: 6: Kinetic effects of trastuzumab for pEGFR and pERK in NCI-N87.
In order to analyze time dependent effects of trastuzumab on the activation of EGFR and ERK in NCI-N87 a kinetic was prepared. Total lysates of untreated cells and cells treated with 5 µg/ml trastuzumab for different time points were analyzed. Untreated samples were prepared at 0, 7, 22 and 72 hours to visualize the effect of the culture time itself. Therefore, the untreated samples were set to 100 % to visualize the effect of the treatment and the culture time. The activation of EGFR and ERK was significantly influenced by trastuzumab. The mean values of three independent experiments are shown with one picture as an exemplary result. Significant effects are indicated by * 0.01 < p-value ≤ 0.05, ** 0.001 < p-value ≤ 0.01 or ***< 0.001 (one-sample and two-sided Welch's t-test).

8 PUBLICATIONS AND CONGRESS CONTRIBUTIONS

8.1 Publications

Keller S*, **Zwingenberger G***, Ebert K, Hasenauer J, Wasmuth J, Maier D, Haffner I, Schierle K, Weirich G, Luber B. Effects of trastuzumab and afatinib on kinase activity in gastric cancer cell lines. Mol Oncol. 2018 Jan 11. doi:10.1002/1878-0261.12170. [Epub ahead of print] PubMed PMID: 29325228.
*both authors contributed equally

Kneissl J, Hartmann A, Pfarr N, Erlmeier F, Lorber T, Keller S, **Zwingenberger G**, Weichert W, Luber B. Influence of the HER receptor ligand system on sensitivity to cetuximab and trastuzumab in gastric cancer cell lines. J Cancer Res Clin Oncol. 2017 Apr;143(4):573-600. doi: 10.1007/s00432-016-2308-z. Epub 2016 Dec 8. PubMed PMID: 27933395; PubMed Central PMCID: PMC5352771.

8.2 Congress contributions

Birgit Luber, Simone Keller, **Gwen Zwingenberger**, Dieter Maier, Birgitta Geier, Fabian Theis, Jan Hasenauer, Sabine Hug, Michael Meyer-Hermann, Azadeh Ghanbari, Axel Walch, Alice Ly, Michaela Aichler, Florian Lordick, Ivonne Haffner. *Identification of predictive response and resistance factors to targeted therapy in gastric cancer using a systems medicine approach*, e:Med Kick-off Meeting on Systems Medicine, 24.-25.11.2014, Heidelberg.

Sabine Hug, Jan Hasenauer, Simone Keller, **Gwen Zwingenberger**, Birgit Luber, Fabian Theis and the SYS-Stomach Consortium. *Multi-level analysis of signalling pathways in gastric cancer data*, e:Med Kick-off Meeting on Systems Medicine, 24.-25.11.2014, Heidelberg.

Birgit Luber, Simone Keller, **Gwen Zwingenberger**, Dieter Maier, Birgitta Geier, Fabian Theis, Jan Hasenauer, Sabine Hug, Michael Meyer-Hermann, Jaber Dehghany, , Axel Walch, Alice Ly, Michaela Aichler, Florian Lordick, Ivonne Haffner. *Identification of predictive response and resistance factors in gastric cancer*, e:Med Meeting 2015 on Systems Medicine 26.-28.10.2015, Heidelberg. (presenting author)

Birgit Luber, Simone Keller, **Gwen Zwingenberger**, Karolin Ebert, Dieter Maier, Birgitta Geier, Fabian Theis, Jan Hasenauer, Sabine Hug, Sabrina Krause, Michael Meyer-Hermann, Jaber Dehghany, Haralampos Hatzikirou, Axel Walch, Michaela Aichler, Florian Lordick, Ivonne Haffner. *Identification of predictive response and resistance factors in gastric cancer*, e:Med Meeting 2016 on Systems Medicine 04.-06.10.2016, Kiel.

Birgit Luber, Simone Keller, **Gwen Zwingenberger**, Karolin Ebert, Dieter Maier, Birgitta Geier, Fabian Theis, Jan Hasenauer, Sabine Hug, Sabrina Krause, Michael Meyer-Hermann, Jaber Dehghany, Haralampos Hatzikirou, Axel Walch, Michaela Aichler, Florian Lordick, Ivonne Haffner. Identification of predictive response and resistance factors in gastric cancer, 28th EORTC-NCI-AACR SYMPOSIUM 29.11.-02.12.2016, München. (presenting author)

J. Kneissl, A. Hartmann, N. Pfarr, F. Erlmeier, T. Lorber, S. Keller, **G. Zwingenberger**, W. Weichert, B. Luber. *Impact of the HER receptor ligand system on the sensitivity to cetuximab and trastuzumab in gastric cancer cell lines*, 101. Jahrestagung der Deutschen Gesellschaft für Pathologie e.V. 22.-24.06.2017, Erlangen.

Elba Raimúndez-Álvarez, Simone Keller, **Gwen Zwingenberger**, Karolin Ebert, Dieter Maier, Birgit Luber, Jan Hasenauer. Understanding the role of HER signaling in resistance to antibody-based therapies in gastric carcinoma using mechanistic models, International Conference on Systems Biology of Human Disease 05.-07.07.2017, Heidelberg.

Simone Keller*, **Gwen Zwingenberger***, Karolin Ebert, Jan Hasenauer, Jacqueline Wasmuth, Dieter Maier, Birgit Luber. *Effects of trastuzumab and afatinib on kinase activity in gastric cancer cell lines*, e:Med Meeting 2017 on Systems Medicine 21.-23.11.2017, Göttingen. (presenting author)
*both authors contributed equally

Elba Raimúndez-Álvarez, Simone Keller, **Gwen Zwingenberger**, Karolin Ebert, Sabine Hug, Dieter Maier, Birgit Luber, Jan Hasenauer. *Modeling the HER pathway in resistance to antibody-based therapies in gastric carcinoma*, e:Med Meeting 2017 on Systems Medicine 21.-23.11.2017, Göttingen.

S. Keller*, **G. Zwingenberger***, K. Ebert, J. Hasenauer, J. Wasmuth, D. Maier, I. Haffner, K. Schierle, G. Weirich, B. Luber. *Effects of trastuzumab and afatinib on kinase activity in gastric cancer cell lines*, 102. Jahrestagung der Deutschen Gesellschaft für Pathologie e.V. 24.-26.05.2018, Berlin. (presenting author)
*both authors contributed equally

S. Keller*, **G. Zwingenberger***, K. Ebert, J. Hasenauer, J. Wasmuth, D. Maier, I. Haffner, K. Schierle, G. Weirich, B. Luber. *Effects of trastuzumab and afatinib on kinase activity in gastric cancer cell lines*, 25th Biennial Congress of the European Association for Cancer Research 30.06.-03.07.2018, Amsterdam (meeting bursary winner). (presenting author)
*both authors contributed equally

Karolin Ebert, **Gwen Zwingenberger**, Simone Keller, Robert Geffers, Dieter Maier, Birgit Luber. *Gene expression analysis of anti-HER therapeutics in gastric cancer cell lines*, e:Med Meeting 2018 on Systems Medicine 24.-26.09.2018, Berlin.

Elba Raimúndez, Simone Keller, **Gwen Zwingenberger**, Karolin Ebert, Sabine Hug, Dieter Maier, Birgit Luber, Jan Hasenauer. *Modeling of the HER signaling pathway in gastric cancer*, e:Med Meeting 2018 on Systems Medicine 24.-26.09.2018, Berlin.