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TESI DI DOTTORATO

PERSONALIZED THERAPEUTIC STRATEGIES AND PREDICTIVE BIOMARKERS TO OVERCOME HER2 RESISTANCE IN GASTRIC CANCER

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ABSTRACT

Gastric cancer (GC) represents one of the most common causes of cancer death worldwide. Surgery is the only curative treatment strategy while conventional chemotherapy and radiotherapy have shown limited efficacy, with a median overall survival of 10 months. Trastuzumab (an epidermal growth factor receptor 2 (HER2) targeting monoclonal antibody), is the only target therapy approved so far in Europe for gastric cancer HER2+ patients with advanced disease (around 22% of incidence). However, only a fraction (<20%) of HER2 amplified patients benefit from treatment, raising doubts about the true cost/effectiveness of this regimen in clinical practice.

At the moment, the best preclinical model to validate molecular targets and positive/negative predictors of response to target therapies is represented by Patient-Derived Xenografts (PDXs), an experimental model that not only retains the principal histologic and genetic characteristics of the donor tumor, but it is also predictive of clinical outcome and is a valuable tool for personalized medicine strategies. We have recently generated a molecularly annotated platform of gastric PDXs (at the moment, >200 PDXs).

The aims of this project were: (i) to identify and validate positive and negative predictors of response to Trastuzumab therapy in gastric cancer and (ii) to establish therapeutic approaches able to overcome Trastuzumab resistance. To reach out these goals, we undertook a prospective evaluation of HER2 targeting with monoclonal antibodies, tyrosine kinase inhibitors and antibody–drug conjugates, in a selected subgroup of HER2 "hyper"-amplified gastric patient-derived xenografts (> 8 HER2 copies), through the design of ad hoc preclinical trials.

Despite the high level of HER2 amplification, Trastuzumab monotherapy elicited a partial response only in 2 out of 8 PDX models. The dual-HER2 blockade with Trastuzumab plus either Pertuzumab or Lapatinib (which have shown better activity than Trastuzumab monotherapy in breast cancer) led to complete and durable responses in 5 (62.5%) out of 8 models, including one tumor bearing a concomitant HER2 mutation. Sequencing data and *in vitro* studies allowed the identification of a HER3 mutation and KRAS amplification as mechanisms of resistance to Trastuzumab therapy. In the resistant PDX harboring KRAS amplification, the recently approved antibody–drug conjugate Trastuzumab deruxtecan overcame KRAS-mediated resistance. We also identified a HGF-mediated non-cell-autonomous mechanism of secondary resistance to

anti-HER2 drugs, responsive to MET co-targeting. These preclinical randomized trials clearly indicate that in HER2-driven gastric tumors, a boosted HER2 therapeutic blockade is required for optimal efficacy, leading to complete and durable responses in most of the cases. Therefore, despite the negative results of previous clinical trials, the dual blockade should be reconsidered for patients with clearly HER2-addicted cancers.

Since, in the last years, cancer-derived extracellular vesicles (EVs) have been regarded as multi-signal messengers in supporting cancer development, progression, and drug resistance, we also explored their role in modulating Trastuzumab sensitivity in HER2 positive gastric cancer cells. We thus (i) evaluated the possibility of using EVs as biomarkers of response to Trastuzumab; (ii) investigated the role of EVs as mediators of resistance.

After a preliminary screening, we identified the presence of the activated HER2 protein only in the EVs of "hyper-amplified" HER2 cell lines. Interestingly, we found that Trastuzumab-induced HER2 inhibition resulted in HER2 dephosphorylation not only in the cells but also in the EVs. In addition, we observed that, *in vitro* and *in vivo*, EVs produced by HER2 positive cells resistant to Trastuzumab were able to render sensitive cells resistant to the drug.

Together, these findings suggest that the analysis of HER2 activation in EVs could be used to monitor the efficacy of HER2 targeted therapies and that transfer of EVs could be one of the mechanisms sustaining resistance to HER2 inhibitors.

INTRODUCTION

1. GASTRIC CANCER: CLINICAL ASPECTS

Although it is steadily declining in incidence, cancer of the stomach (also known as gastric cancer (GC)) remains one of the most common and deadly neoplasms in the world (1). According to GLOBOCAN 2018 data, gastric cancer is the third leading cause of cancer deaths worldwide, following only lung and colorectal cancer in overall mortality. About 1 in 12 of all oncological deaths are attributable to gastric cancer. Over a million new cases of gastric cancer are diagnosed, worldwide, each year (1).

1.1 EPIDEMIOLOGY

Stomach cancer is the 5th most commonly diagnosed cancer in the world, and the 7th most prevalent.

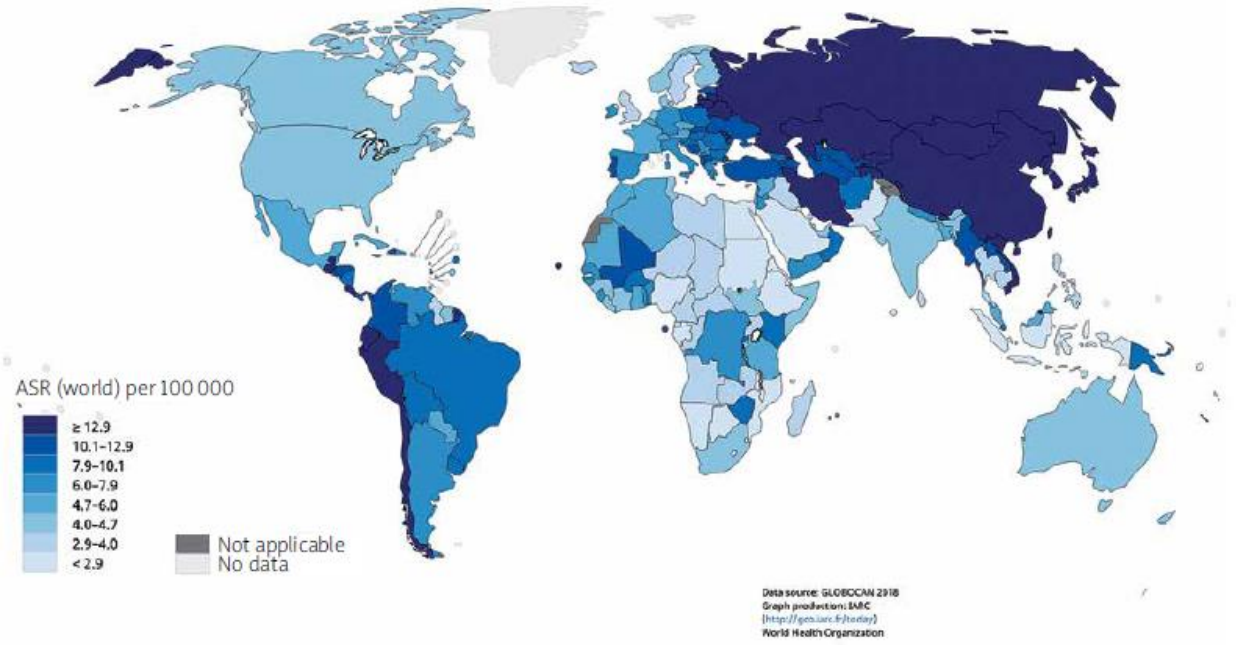


Figure 1: Map shows the estimated age-standardized incidence rates (world) for stomach cancer in 2018, both sexes, all ages. Reproduced from http://globocan.iarc.fr.

The cumulative risk of developing gastric cancer from birth to age 74 is 1.87% in males and 0.79% in females worldwide (1). In developed countries, gastric cancer is thus 2.2 times more likely to be diagnosed in males than females. In developing countries, this ratio is 1.83 (1).The incidence of gastric cancer is highly variable by region and culture. Incidence rates are highest in Eastern and Central Asia and Latin America where 8.3% of all cancer deaths are attributable to gastric cancer (Figure 1) (2). The cumulative risk of death from gastric cancer, from birth to age 74, is 1.36% for males and 0.57% for females (1). Mortality rates are high in eastern and central Asia and Latin America, the same regions with high incidence (Figure 2).

For males, gastric cancer is the leading oncological cause of death in 10 nations worldwide, specifically, eastern, and central Asian. For females, it is the leading oncological cause of death in 4 nations.

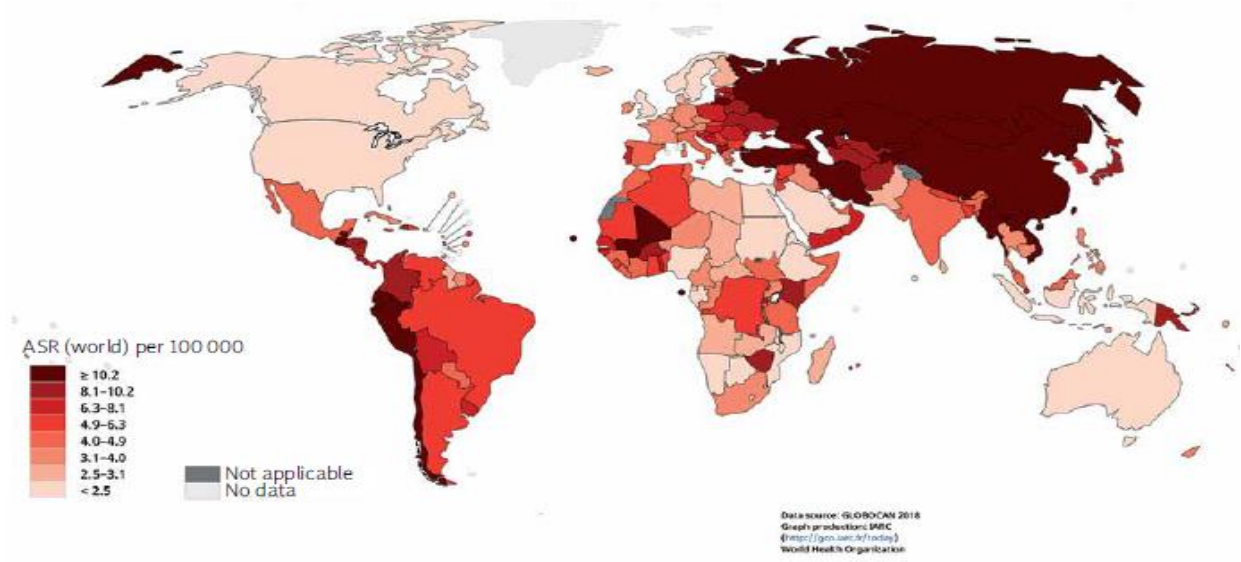


Figure 2: Map shows the estimated age-standardized mortality rates (world) for stomach cancer in 2018, both sexes, all ages. Reproduced from http://globocan.iarc.fr.

1.2 GENETICS

The major part of gastric cancers is sporadic, while 10% is hereditary; at least three familial syndromes exist including gastric cancer: hereditary diffuse gastric cancer (HDGC), gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS) and familial intestinal gastric cancer (FIGC). HDGC is caused by an autosomal dominant germline mutation in the CDH1 gene that encodes for E-cadherin. This protein is involved in cell-to- cell adhesion and its loss of function contributes to neoplastic progression and facilitates tumor metastases through spread of cancer cells across the tissue basement membrane. This condition predisposes patients to both early onset diffuse gastric cancer and lobular breast cancer (3). Prophylactic total gastrectomy at a center of expertise is recommended for individuals with pathogenic CDH1 mutations (4). GAPPS, first described in 2012, is characterized by autosomal dominant transmission of fundic gland polyposis (including dysplastic areas or adenocarcinoma or both) restricted to the proximal stomach with no evidence of colorectal/duodenal polyposis or other hereditary gastrointestinal cancer syndromes (5). FIGC is also inherited with an autosomal dominant pattern but, unlike GAPPS, there is no evidence of gastric polyposis. The genetic alteration has not yet been identified neither for GAPPS nor for FIGC (3). Gastric cancer is also linked to a range of cancer-associated syndromes with known genetic causes, such as Lynch, Li-Fraumeni, Peutz-Jeghers, hereditary breast-ovarian cancer syndromes, familial adenomatous polyposis, and juvenile polyposis (6).

1.3 RISK FACTORS

Numerous risk factors have been recognized for the onset of gastric cancer: they include genetics, consumption of high salt and nitrite-containing foods, smoking, obesity, Helicobacter pylori infection (H. pylori), pernicious anemia, and chronic atrophic gastritis (figure 3) (3). It has been demonstrated that some combinations of single nucleotide polymorphisms (SNPs) in immune-related genes (interleukin 1-b, interleukin 1 receptor antagonist, tumor necrosis factor and interleukin 10) increase the risk of developing gastric cancer, but only in H. pylori infected patients.

Figure 3: Gastric cancer risk factors. Modified from http://learnaboutcancer.net

About 10% of gastric cancers are Epstein-Barr virus (EBV) positive. EBV may directly contribute to the development of EBV-associated gastric cancer. This tumor-promoting effect seems to involve multiple mechanisms, because EBV affects several host proteins and pathways that normally promote apoptosis and regulate cell proliferation. EBV-associated gastric carcinomas have some distinctive clinic-pathological characteristics: they occur predominantly in men and in younger individuals and generally present a diffuse histological type. Furthermore, most cases exhibit rich lymphocyte infiltration.

1.4 CLINICAL PRESENTATION AND DIAGNOSIS

Gastric cancer is often diagnosed in advanced stages, mainly because it does not present characteristic clinical features or pathognomonic symptoms. Vague abdominal pain, nausea and dyspepsia may be associated with weight loss, early satiety, or melena as the disease progresses. Gastric tumors preferentially present a local invasion that can lead to external compression and distal obstructive symptoms, gastro-intestinal bleeding, colonic invasion, and subsequent obstruction, or gastric perforation. Lymph nodes, liver, and the peritoneal surface are the most common sites of metastasis. The gold standard for gastric cancer diagnosis is EGD (Esophagogastroduodenoscopy) because it allows direct visualization of the disease. Furthermore, biopsies can be performed for histological confirmation and to identify any precancerous lesions or H. pylori infection (3). Even though EGD is excellent for recognizing developed or large tumor masses, it lacks sensitivity when identifying early lesions. For this reason, some new techniques have been introduced, such as high- resolution endoscopy with narrow band imaging and image-enhanced endoscopy, in order to support the identification of microscopic lesions (7). EUS (Endoscopic Ultrasound) is also a useful tool, particularly to assess tumor and nodal staging, allowing biopsies of suspected and endoscopically accessible lymph nodes (3). CT and FDG-PET are required for disease staging. CT scan is usually performed with IV contrast and allows a description of tumor growth, local invasion, and metastases, whereas FDG-PET is important to detect metastases and to assess tumor biology and response (3).

1.5 THERAPEUTIC APPROACHES IN LOCALIZED DISEASE

Randomized clinical trials provide evidence that combined modality therapy is effective for patients with non-metastatic gastric and gastroesophageal adenocarcinoma. Perioperative chemotherapy or postoperative chemotherapy plus chemoradiation are listed as preferred

approaches in current guidelines, although postoperative chemotherapy is also an option after an adequate lymph node dissection. (8)

A relatively new development in the workup of patients with potentially resectable disease is microsatellite instability (MSI) testing at diagnosis. There are several studies suggesting that patients with MSI-high cancers may have an adverse oncologic outcome when treated with standard systemic chemotherapy approaches (9). In a secondary post hoc analysis of the MAGIC trial, patients with MSI-high tumors had improved survival with surgery alone and inferior survival with perioperative chemotherapy plus surgery compared with those having microsatellite-stable tumors (10).

1.6 TREATMENT OF METASTATIC AND UNRESECTABLE GASTRIC CANCER

Several cytotoxic agents are active in advanced gastric cancer, including fluoropyrimidines, platinums, taxanes, and irinotecan. The choice of treatment depends on patient performance status and medical comorbidities as well as the toxicity profile of the regimen. Combination regimens offer higher response rates and improved survival compared with single-agent therapy. Treatment goals are typically palliative in intent and are aimed at controlling symptoms, controlling disease, and extending life. Although there is no universal standard first-line therapy, a fluoropyrimidine and platinum doublet is typically the preferred backbone regimen for most patients. Oxaliplatin is considered to be as effective as cisplatin and is the choice platinum in most modern regimens (11).

In patients with overexpression or amplification of HER2, Trastuzumab should be added to cytotoxic first-line chemotherapy, as reviewed in detail in the third paragraph. In patients with a programmed cell death ligand 1 (PD-L1) combined positive score (CPS) ≥5, nivolumab should be added to first-line chemotherapy (12).

In the second-line treatment for metastatic gastric cancer, cytotoxic chemotherapy agents not already used in the first line can be attempted. Several years ago, ramucirumab was added to the armamentarium of active agents in this disease. Ramucirumab is a monoclonal antibody that binds to VEGF receptor-2 (VEGFR-2), blocking receptor activation. In the phase 3 REGARD trial ramucirumab was shown to elicit a 1.4-month survival benefit compared with placebo in the second-line treatment of advanced gastric adenocarcinoma (13). Subsequently, the phase 3 RAINBOW trial demonstrated that paclitaxel plus ramucirumab was superior to paclitaxel plus placebo in the second line setting with an OS of 9.6 versus 7.4 months (14).

2. CLASSIFICATION SYSTEMS

Stomach cancer refers to any malignant neoplasm that arises from the region extending between the gastroesophageal junction and the pylorus. Roughly 95% of stomach tumors are of epithelial origin and are classified as adenocarcinomas. Histological types, such as adenosquamous, squamous, and undifferentiated carcinomas are rare (15). Several classification systems are in place for gastric cancer based on histology. The most used are Lauren's classification and the WHO system (3). Since these types of classification do not have a prognostic or predictive role, they cannot be used to guide the clinical management of the disease, neither initially for potentially curative treatment, nor palliatively, for advanced disease (16). In order to supply this need, The Cancer Genome Atlas (TCGA) Research Network and the Asian Cancer Research Group (ACRG) proposed two different but related classifications, based on molecular profiling data.

2.1 LAUREN'S CLASSIFICATION

This system describes two main subtypes of gastric adenocarcinoma: intestinal and diffuse (Figure 4) (17). The intestinal subtype is more common in men and older people in high- risk regions and is characterized by a better prognosis (15). It is often associated with H. Pylori infection and is defined as the last step after a precancerous cascade which consists of i) non-atrophic gastritis, which is the result of acute inflammation with infiltration of polymorphonuclear cells within the gastric glands; ii) atrophic gastritis with a loss of parietal cells; iii) intestinal metaplasia in which injured gastric cells originate glands with intestinal phenotype; iv) dysplasia, that is characterized by nuclear atypia and architectural disorganization (3).

The diffuse subtype may also be associated with H. Pylori, but precancerous lesions are not well defined (3). It is the predominant subtype in endemic areas, in which the incidence is low, and it is more frequent in women and younger patients (15).

The pathognomonic feature of this subtype is the presence of signet ring cells (18), whose name is due to their singular appearance: the nucleus is displaced to the periphery by intracellular mucin. These cells lose the expression of E-cadherin, that plays an important role in cell adhesion, and they are prone to infiltrate through the stomach wall, which thus results leathery and thickened, giving rise to *linite plastica* (19).

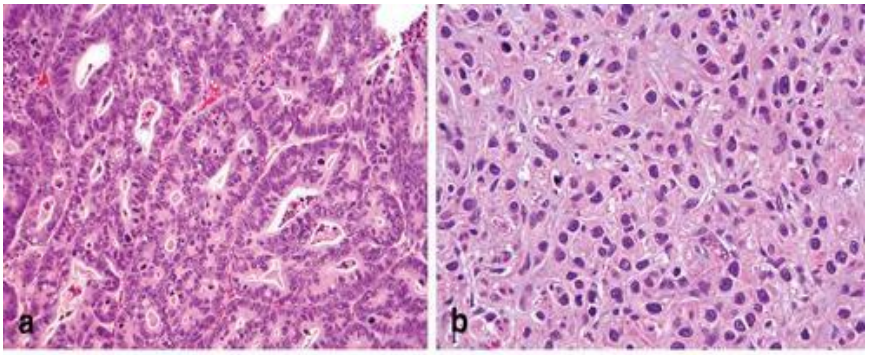


Figure 4: Main histological subtypes according to Lauren's classification.
a. Intestinal subtype, with well-formed glandular and tubular architecture.
b. Diffuse subtype, poorly differentiated.
Adapted from https://abdominalkey.com/p

2.2 WHO CLASSIFICATION

WHO classification recognizes six subtypes according to their predominant histological pattern: I) papillary adenocarcinoma, with elongated, finger-like processes supported by fibrovascular stalks and lined by cuboidal or cylindrical cells, II) tubular adenocarcinoma, with tubules of irregular shapes and sizes, lined by cuboidal, cylindrical or flattened cells, III) mucinous adenocarcinoma, in which mucin is the major component of the extracellular matrix and fills the gland lumen, compressing the epithelium and forming mucin lakes where cells float, IV) signet-ring cell carcinoma, in which mucin is intracellular, and other less frequent subtypes, such as V) poorly cohesive carcinoma and VI) undifferentiated carcinoma (20) (Figure 5).

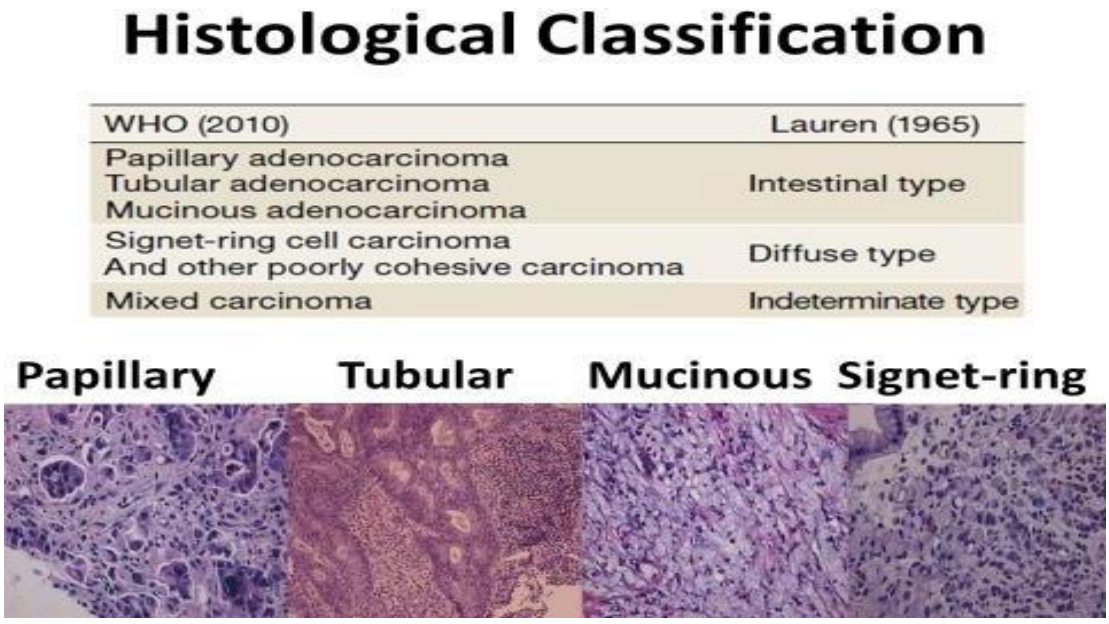


Figure 5: Main histological subtypes according to WHO classification. Adapted from https://abdominalkey.com/p

2.3 MOLECULAR CLASSIFICATION

Very recently, new classifications have been proposed on the basis of the molecular landscape of gastric cancer, such as those of The Cancer Genome Atlas (TCGA) Research Network and of the Asian Cancer Research Group (ACRG) (21) (22) .

The TCGA group performed a comprehensive molecular characterization of gastric tumors from 295 patients who had not been treated whit prior chemotherapy or radiotherapy. They used germline DNA from blood or non-malignant gastric mucosa as a reference for detecting somatic alterations and they characterized samples using six molecular platforms: array-based somatic copy number analysis, whole exome sequencing, array-based DNA methylation profiling, messenger RNA sequencing, microRNA sequencing and reverse- phase protein array (RPPA). Moreover, microsatellite instability testing was performed on all tumors and low-pass whole genome sequencing on 107 tumor/germline pairs (21). The integrated analysis of all these platforms allowed the identification of four molecularly distinct subtypes (Figure 6).

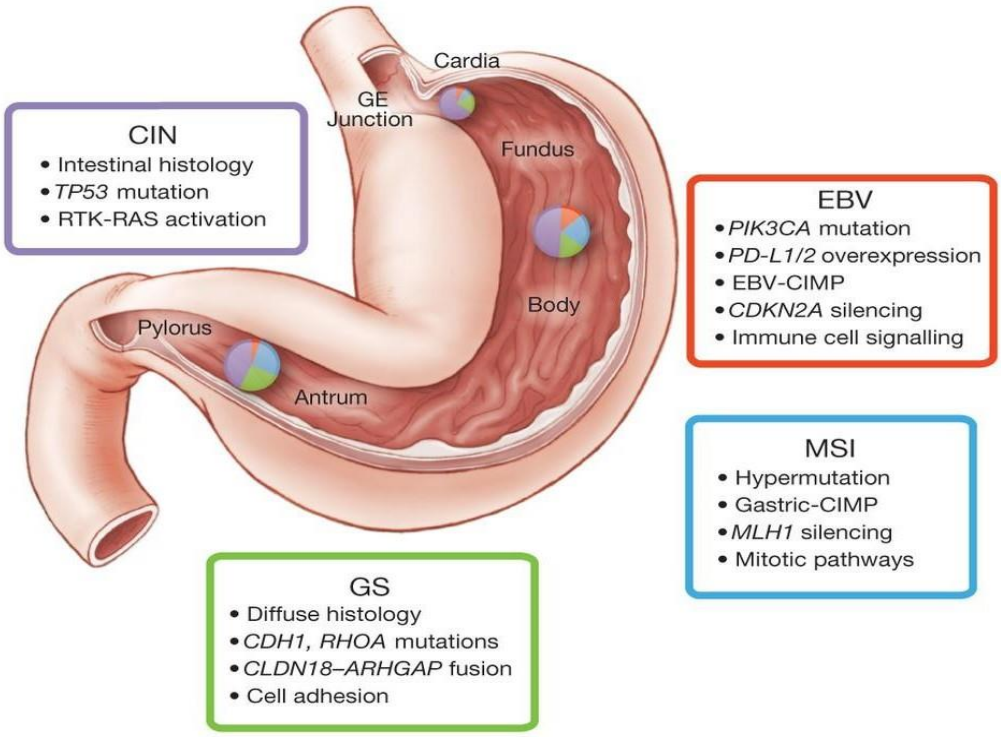


Figure 6: Key features of gastric cancer subtypes. This scheme lists some of the salient features associated with each of the four molecular subtypes of gastric cancer. Distribution of molecular subtypes in tumors obtained from distinct regions of the stomach is represented by inset charts. Adapted from "Comprehensive molecular characterization of gastric adenocarcinoma" (21). Details are given in the text.

The first group of tumors was significantly enriched for EBV burden and showed extensive DNA promoter hypermethylation, higher than any other tumor reported by the TCGA. EBV status was determined using mRNA, miRNA, exome and whole genome sequencing. All EBV-positive tumors displayed CDKN2A promoter hypermethylation and 80% had PIK3CA mutations. In addition, PD-L1/2 expression was elevated in EBV-positive tumors, predicting a role of targeted immunotherapy in this subset of gastric tumors. Other alterations commonly found were mutations in ARID1A (55%) and BCOR (23%) and amplification of JAK2 (15%).

The second group was enriched for MSI (Micro Satellite Instability, 22% of gastric adenocarcinomas) and showed elevated mutation rates and hypermethylation (including at the MLH1 promoter). Mutations of kinases such as EGFR (5%), HER2 (5%), HER3 (14%), JAK2 (11%), FGFR2 (2%), MET (3%), and PIK3CA (42%) were also present.

The third group, named genomically stable (GS), was enriched for the diffuse histological

variant and has newly described mutations in RHOA, which acts through several effectors to control actin/myosin-dependent cell contractility and motility. In addition, interchromosomal translocation (between CLDN18 and ARHGAP26, the latter implicated in cell motility) was found in genomically stable gastric tumors; moreover, mutations of CDH1 were present in 26 % of the cases. Genomically stable tumors were diagnosed at an earlier age.

The last group represented almost half of gastric tumors and was characterized by chromosomal instability (CIN), marked aneuploidy and focal amplification of receptor tyrosine kinases and KRAS; moreover, amplification of VEFGA and of cell cycle mediators (CCNE1, CND1, and CDK6) was quite frequent. The frequency of p53 mutations was the highest among the microsatellite stable groups.

Based upon these results, the TCGA group created a decision tree to categorize the 295 gastric cancer samples in the four subtypes (Figure 7) using an approach that could readily be applied to gastric cancer tumor in clinical care. Tumors were first categorized by EBV-positivity (9%), then by MSI-high status, hereafter called MSI (22%), and the remaining tumors were distinguished by the degree of aneuploidy into those termed genomically stable (20%) or those exhibiting chromosomal instability (CIN 50%).

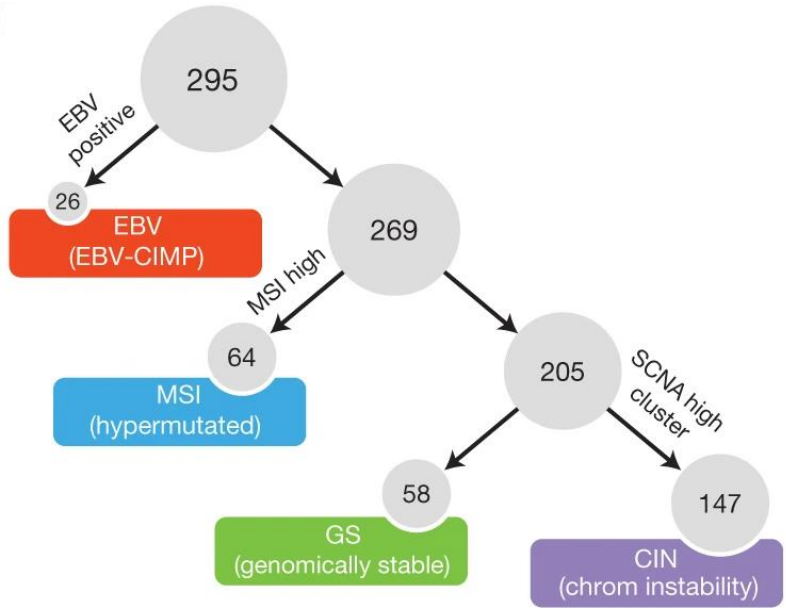


Figure 7: A flowchart outlines how tumors were classified into molecular subtypes. Adapted from "Comprehensive molecular characterization of gastric adenocarcinoma" (38).

Through the study of the molecular and genomic basis of gastric cancer, the researchers described a molecular classification that may serve as a valuable adjunct to histopathology. Importantly, these molecular subtypes showed distinct salient genomic features, providing a guide to the use of targeted agents that should be evaluated in clinical trials for distinct populations of gastric cancer patients. However, since the authors of this work did not observe survival differences among the four subgroups, they concluded that this classification is not endowed with prognostic value. However, in the following years, the TCGA classification has become the cornerstone of many publications that have exploited the proposed subtypes in different cohorts of gastric cancer patients. Starting from the TCGA classification, Sohn and colleagues demonstrated the clinical significance of the 4 gastric cancer subtypes and developed predictive models that can reliably stratify gastric cancer patients. Their predictive model can be used to identify not only patients with a poor prognosis (GS subtype), but also those who would benefit most from adjuvant chemotherapy (CIN subtype) (23). Lan Q et al., instead, used the TCGA database and the ESTIMATE algorithm to obtain a list of genes related to the tumor microenvironment that predicts poor prognosis in GC patients (24). The functions of these genes were further validated in another independent GC cohort (GEO).

The second study was carried out by the Asian Cancer Research Group who provided a new classification of gastric cancer based on the analysis of 300 tumors. The authors performed gene expression profiling, genome-wide copy number microarrays, targeted gene sequencing and defined four distinct GC molecular subtypes associated with distinct genomic alterations, survival outcome and recurrence patterns after surgery (22).

In figure 8 it is reported a decision tree to categorize gastric cancer samples according to ACRG: of the 300 tumors examined, 68 displayed microsatellite instability while 46 of the other 232 showed epithelial mesenchymal transition (MSS/EMT). The remaining samples differed for the status (active or inactive) of p53 (MSS/TP53+ or MSS/TP53-).

The MSS/EMT subtype occurred at significantly younger age, was mainly of the diffuse type (>80% of the cases) and was mainly diagnosed at stage III/ IV; it showed the worst prognosis, had the highest chance of recurrence (mostly peritoneal) and a lower number of mutations.

The MSI subtype occurred mainly in the antrum (75%), >60 % of the tumors were of intestinal subtype, and >50% of subjects were diagnosed at an early stage (I/II). Among the different types, the MSI group showed the best prognosis, the lowest chance of recurrence (mostly liver) and the presence of hypermutation, with mutations in genes such as KRAS (23,3%), the PI3K-PTEN- mTOR pathway (42%), ALK (16,3%) and ARID1A (44,2%).

The MSS/TP53+ had an intermediate prognosis and intermediate recurrence. Epstein-Barr virus infection occurred more frequently in this group and there was a high frequency of mutations in APC, ARID1A, KRAS, PIK3CA and SMAD4. The last group had characteristics similar to MSS/TP53+ but it showed p53 mutations and genomic instability (focal amplifications of HER2, EGFR, CCNE1, CCND1, MDM2, ROBO2, GATA6, MYC and chromosome-wide copy number variation).

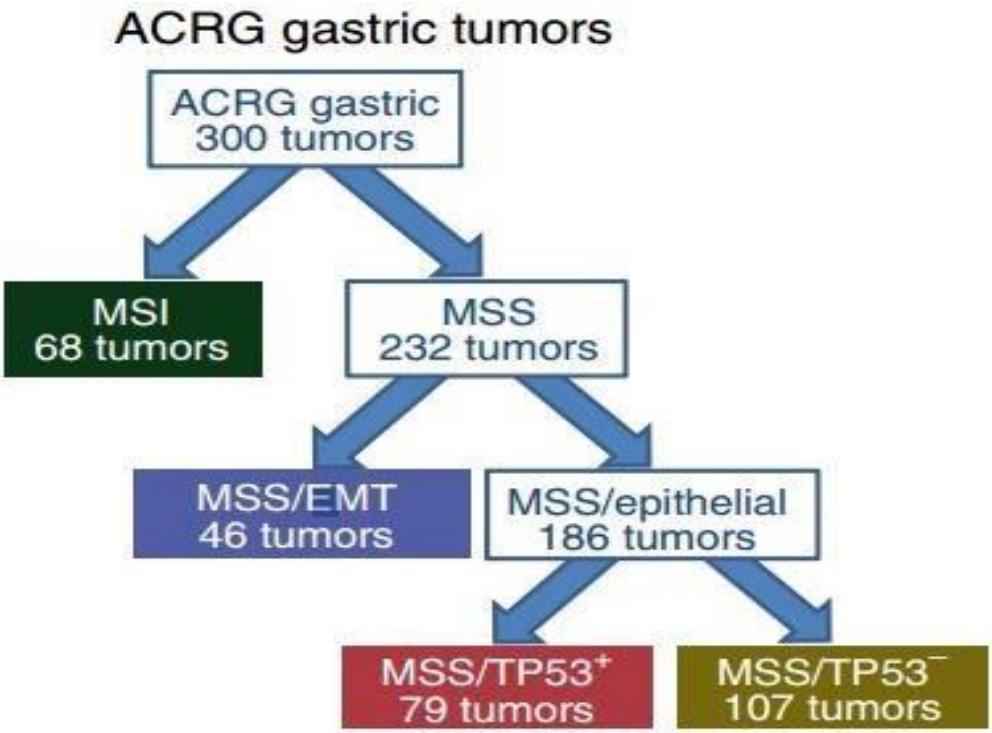


Figure 8: A flowchart outlines how tumors were classified into molecular subtypes. Adapted from "A new molecular classification of gastric cancer proposed by Asian Cancer Research Group" (22).

This new molecular classification is produced based on a large sample set and shows a good repeatability. It provides a good paradigm, especially for Chinese researchers to establish new molecular classification of gastric cancer in Chinese cohorts. However, there are some critical points in this publication. First, the molecular classification system is mainly based on gene expression profiles, and the data from whole genome sequencing or whole transcriptomic sequencing are poor. Second, regarding to the recurrence of gastric cancer, the endpoint of recurrence was not strict enough, because the biological significance of recurrence time is different (1-year recurrence, 2-year recurrence or 5-year recurrence post-operation). Finally, the clinicians are expecting to get more guidance for treatment from the knowledge of the different molecular subtypes. Comparison of both classifications indicates the presence of similarities but also differences between them. The MSI subtype was found in both classifications and characterized by high mutation frequency and the best prognosis. Afterwards, TCGA marked out also GS, EBV+ and CIN subtypes, while ACRG classification described MSS/EMT, MSS/TP53+ and MSS/TP53− GCs subtypes. When comparing the TCGA chromosomal unstable group and the ARCG MSS/TP53- group it was notable that TCGA chromosomally unstable subtype comprised a higher fraction of the total number of tumors than the TP53 negative subgroup. CIN and GS TCGA tumors were present across all ACRG types. The difference in frequency of CDH1 and RHOA mutation results in a TCGA GS class that is not equivalent to the ACRG MSS/EMT subtype. Furthermore, the TCGA GS subtype was not equivalent with the ACRG MSS/EMT subtype either and the MSS/TP53+ group did not overlap with the TCGA EBV subtype. Possible reasons for these differences could be the larger proportion of Laurén diffuse type GCs in the ACRG group (24% in TCGA versus 45% in ACRG) and lesser tumors were located proximally and at the GE junction. Also, the ethnic origin of the patients was different (USA and Western Europe vs. Korea). Finally, different platforms for genetic studies in the two projects were used. Associations between genetic aberrations of cancer-related genes and clinical outcomes were studied in Korean GC patients. Researchers have compared both classifications, TCGA and ACRG, with patients outcomes. Analysis of clinical outcomes by using both classifications showed that EBV group had the best survival.

3. THE RECEPTOR TYROSINE-PROTEIN KINASE ERBB-2 (HER2)

The human epidermal growth factor receptor (HER) family of receptors plays a central role in the pathogenesis of several human cancers. They regulate cell growth, survival, and differentiation via multiple signal transduction pathways and participate in cellular proliferation and differentiation. The family is made up of four members: HER-1, HER- 2, HER-3, and HER-4 (25). All four HER receptors comprise a cysteine-rich extracellular ligand binding site, a transmembrane lipophilic segment, and an intracellular domain with tyrosine kinase catalytic activity (26). Epidermal growth factor receptor (EGFR, ErbB1 or HER1) was the first receptor tyrosine kinase discovered by Carpenter and co-workers at Vanderbilt University, USA, in 1978 (27). ErbB stands for its homology with the Erb-b gene responsible for avian erythroblastosis (28). The neu oncogene (also known as HER2, ErbB2, or P185) was discovered by a group of scientists at Massachusetts Institute of Technology, Rockefeller, and Harvard University (29) (30). The HER2 receptor is a 1255 amino acid, 185 kD transmembrane glycoprotein located at the long arm of human chromosome 17 (17Q12) (31).

3.1 FUNCTION

HER receptors exist as monomers on the cell surface. Upon binding to their ligands through the extracellular domains, HER proteins undergo dimerization and transphosphorylation of their intracellular domains. HER2 has no known direct activating ligand and is in a partially activated state constitutively, becoming fully active upon heterodimerization with other family members such as HER1 and HER3. Homo or heterodimerization result in the autophosphorylation of tyrosine residues within the cytoplasmic domain of the receptors and initiate a variety of signaling pathways, principally the mitogen-activated protein kinase

(MAPK), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), and protein kinase C (PKC) resulting in cell proliferation, survival, differentiation, angiogenesis, and invasion. Heterodimers generate more potent signals than homodimers, and those containing HER2 have a particularly signaling potency as HER2 exists in an open conformation, making it the dimerization partner of choice among the family members. The HER2-HER3 heterodimer is the most potent stimulator of downstream pathways, particularly the PI3K/Akt one, a master regulator of cell survival (32). Moreover, HER2 dimerization promotes the mislocalization and rapid degradation of the cell-cycle inhibitor P27KiP1 protein, leading to cell-cycle progression (33) (34) (35). HER2 can also be activated by complexing with other membrane receptors such as insulin-like growth factor receptor 1 and plexin B1 (36). Figure 9 shows the main transduction pathways regulated by the four HER family members—EGFR, HER2, HER3, and HER4 (37).

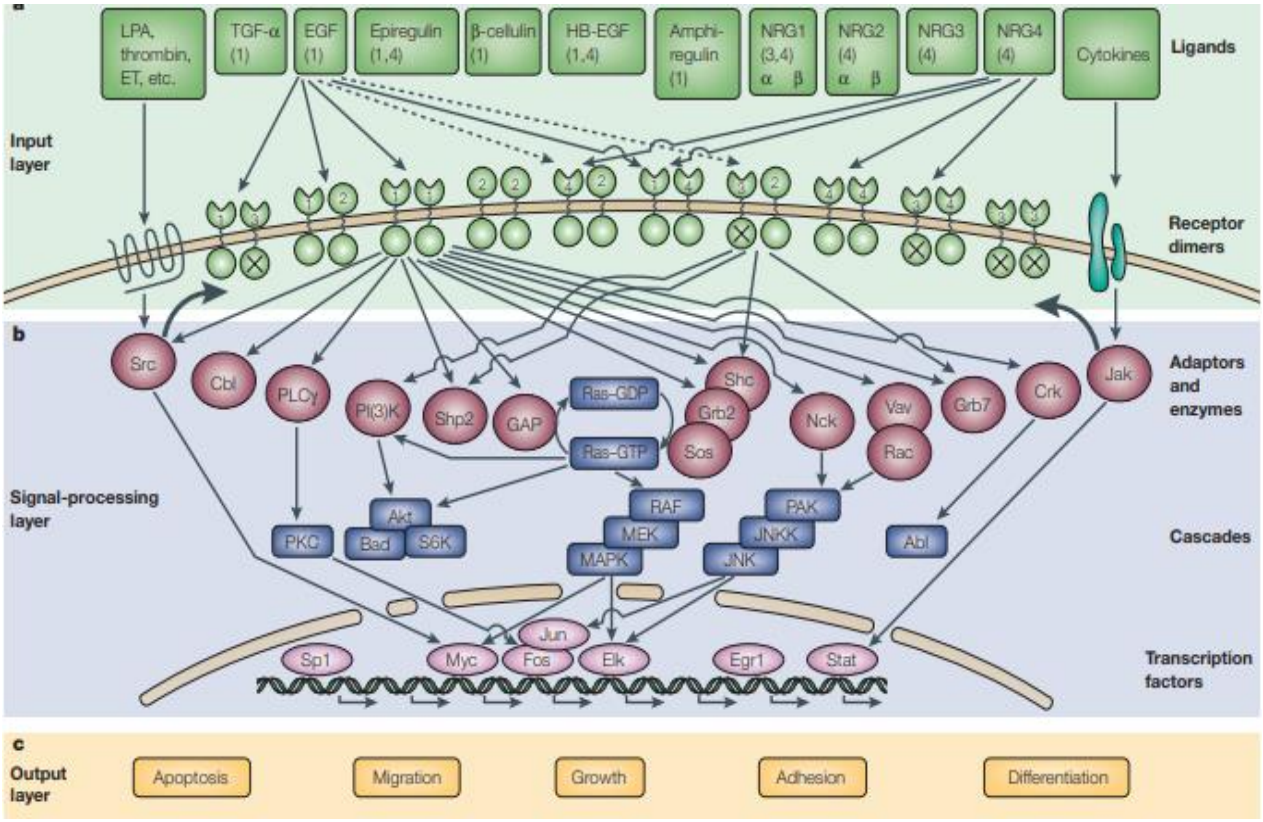


Figure 9: The ErbB signaling network. **a** | Ligands and the ten dimeric receptor combinations comprise the input layer. Numbers in each ligand block indicate the respective high-affinity ErbB receptors. For simplicity, specificities of receptor binding are shown only for epidermal growth factor (EGF) and neuregulin 4 (NRG4). ErbB2 binds no ligand with high affinity, and ErbB3 homodimers are catalytically inactive (crossed kinase domains). *Trans*-regulation by G-

protein-coupled receptors and cytokine receptors is shown by wide arrows. **b** | Signaling to the adaptor/enzyme layer is shown only for two receptor dimers: the weakly mitogenic ErbB1 homodimer, and the relatively potent ErbB2–ErbB3 heterodimer. Only some of the pathways and transcription factors are represented in this layer. **c** | How they are translated to specific types of output is poorly understood at present. (Abl, a proto-oncogenic tyrosine kinase whose targets are poorly understood; Akt, a serine/threonine kinase that phosphorylates the anti-apoptotic protein Bad and the ribosomal S6 kinase (S6K); GAP, GTPase activating protein; HB-EGF, heparin-binding EGF; Jak, janus kinase; PKC, protein kinase C; PLCγ, phospholipase Cγ; Shp2, Src homology domain-2-containing protein tyrosine phosphatase 2; Stat, signal transducer and activator of transcription; RAF–MEK–MAPK and PAK–JNKK–JNK, two cascades of serine/threonine kinases that regulate the activity of a number of transcription factors). Adapted from "Untangling the ErbB signaling network" (38).

3.2 HER2 OVEREXPRESSION IN GASTRIC CANCER

Most of the studies on HER2 have been carried out in breast cancer, after it was found to induce mammary carcinogenesis in vitro (39) and in vivo (40). Amplification or overexpression of the HER2 gene occurs in approximately 15–30% of breast cancers (41). With increasing understanding of HER2 biology, it has now been recognized that HER2 overexpression also occurs in other forms of cancers such as stomach, ovary, uterine serous endometrial carcinoma, colon, bladder, lung, uterine cervix, head and neck, and esophagus (42) (43). Apart from its role in development of various cancers, it has also been intensely evaluated as a therapeutic target.

HER2 in Gastric Cancer. Overexpression of HER2 protein in gastric cancer, using immunohistochemistry (IHC), was first described in 1986 (44). Although some small-scale studies have not demonstrated the prognostic properties of HER2 (45) (46) (47), a larger number of studies indicate that HER2 amplification is a negative prognostic factor, showing more aggressive biological behavior and higher frequencies of recurrence (48) (49). HER2 overexpression in patients with gastric cancer has been reported from 10 to 30% and correlates with poor outcome and a more aggressive disease (50).

In a study by Yano, HER2 overexpression by IHC was found in 23% and gene amplification by FISH in 27% of 200 resected tumors (51). Gravalos and Jimeno in their study of 166 gastric cancer

patients observed that HER2 overexpression was most commonly found in gastroesophageal junction (GEJ) tumors and tumors having intestinal type histology (52). Other studies also confirmed a higher rate of HER2 positivity in GEJ tumors and intestinal subtype (53) (54).
In a study of 260 gastric cancers, HER2 overexpression was an independent negative prognostic factor and HER2 staining intensity was correlated with tumor size, serosal invasion, and lymph node metastases (55).

HER2 in Esophageal Cancer. HER2 overexpression is reported in 0–83% of esophageal cancers, with a tendency towards higher rates of positivity in adenocarcinoma (10–83%) compared to squamous cell carcinomas (0–56%) (56) (57) (58). Yoon et al., in their study of 713 patients with surgically resected esophageal adenocarcinomas (EAC), found HER2 positivity in 17% of patients, significantly associated with lower tumor grade, less invasiveness, fewer malignant nodes, and the presence of adjacent Barrett's esophagus (59).

Barrett's Esophagus (BE). In EACs with Barrett's esophagus, HER2 positivity was significantly associated with improved DSS [HR = 0.54 (95% CI: 0.35–0.84), P = 0.0065] and overall survival (P = 0.0022) independent of pathologic features but was not prognostic among EACs without BE. However, another study by the same authors found that HER2 heterogeneity among HER2 amplified EACs was an independent predictor of worse cancer-specific survival (60). Apart from EAC, HER2 overexpression was also found to be a negative predictor of survival in esophageal squamous cell carcinoma (61).

3.3 TESTING FOR HER2

Although several methods for HER2 testing have been developed, approximately 20% of current HER2 testing may be inaccurate. Therefore, the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) have recommended guidelines in HER2 testing to ensure accuracy (62). The two methods currently approved for HER2 testing are immunohistochemistry (IHC) and in situ hybridization (ISH).

In gastric cancers, the heterogeneity of the HER2 genotype can lead to discrepancies in the results from IHC and FISH testing (63). Tumor heterogeneity was seen in roughly 4.8% of samples with moderate or strong HER2 staining and was higher than what was experienced in breast cancer (1.4%) (64). ASCO/CAP guidelines state that intratumoral heterogeneity may contribute to HER2 testing inaccuracy. Incomplete basolateral membrane HER2 IHC staining is also more common in gastric cancer than in breast cancer. This is due to the higher frequency of glandular formations that occur in gastric tissue. Moreover, in gastric tissue, the basolateral membrane is stained, not the luminal membrane, resulting in the heterogeneity. Currently, there are no ASCO/CAP approved HER2 testing guidelines for gastric cancer. The National Comprehensive Cancer Network (NCCN) guidelines panel recommended that Trastuzumab should be offered to HER2-positive gastric cancer patients (Figure 1). Immunohistochemistry (IHC) and in situ hybridization (FISH) analysis are required to assess HER2 positivity. A strong, complete, basolateral or lateral membranous staining in ≥ 10% of tumor cells (in surgical specimens) and a tumor cell cluster with a strong, complete, basolateral or lateral membranous reactivity (in biopsy specimens) are defined as IHC strongly positive (IHC3+): this is sufficient to consider a sample as HER2 positive. IHC0/1+ is considered negative, whereas IHC2+ requires ISH assessment.

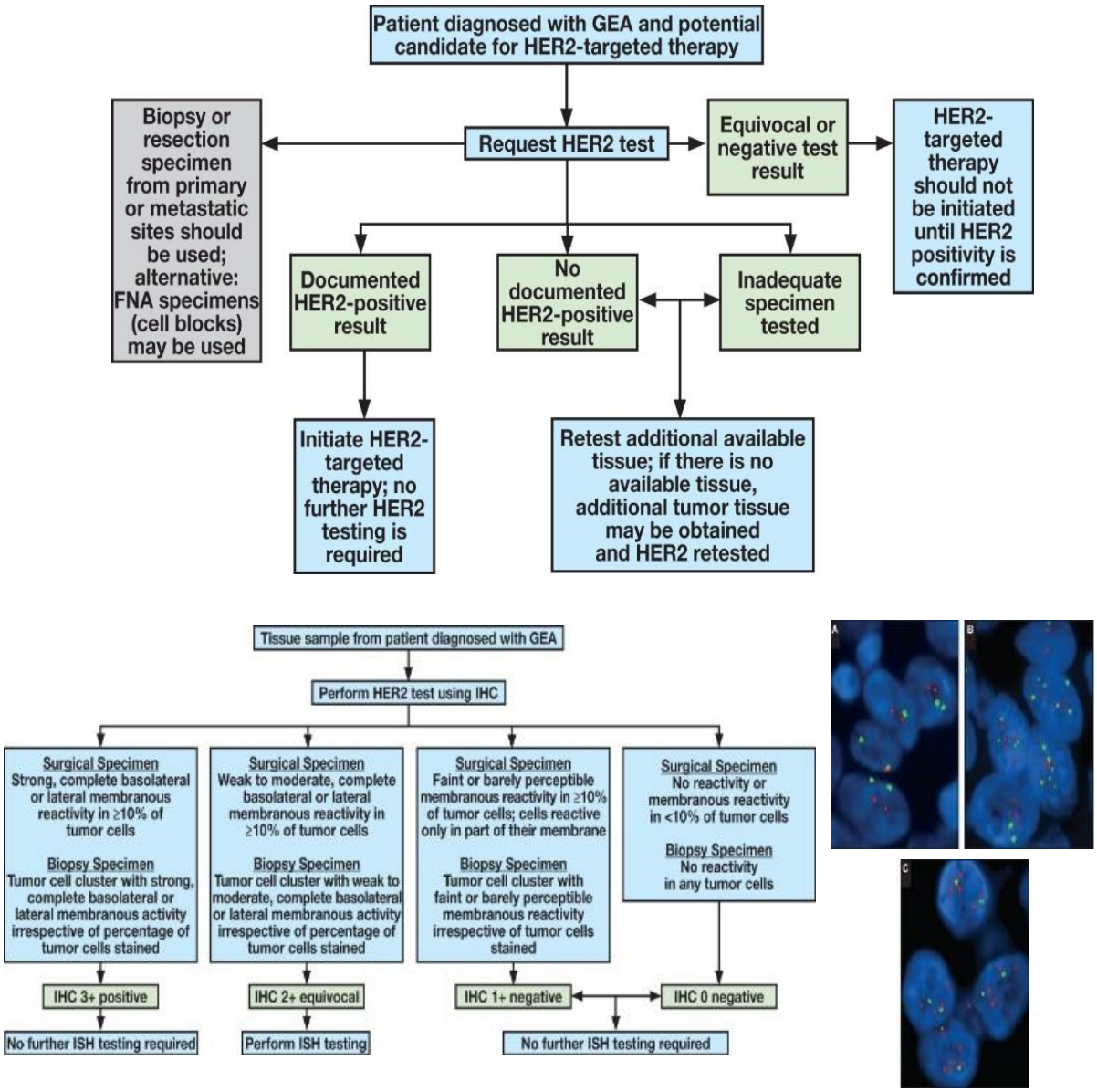


Figure 10: Flow chart to assess HER2 positivity in gastric cancer. GEA: Gastro-Esophageal Adenocarcinoma. FNA: Fine Needle Aspiration. IHC: Immunohistochemistry. Modified from "HER2 Testing and Clinical Decision Making in Gastroesophageal Adenocarcinoma: Guideline From the College of American Pathologists, American Society for Clinical Pathology, and American Society of Clinical Oncology" (65).

3.4 HER2 TARGETING IN GASTRIC CANCER

In the last decade we have witnessed the development of an impressive number of HER2 targeted therapies, many of them being investigated in GC patients. However, since the publication of the pivotal ToGA trial which established the combination of fluoropyrimidine-cisplatin plus Trastuzumab as the standard of care in treatment-naïve patients with HER2-positive metastatic GC, a plethora of negative trials on HER2 targeted therapies in GC patients have been presented or published (53); in fact, the LOGiC, TyTAN, JACOB, and GATSBY trials on Lapatinib, Pertuzumab, and Trastuzumab emtansine (T-DM1) failed to meet their primary endpoints (66) (67) (68) (69) (Table 1). More recently, we have seen the development of novel and promising HER2 targeted treatments as monotherapy or in combination with other anticancer agents, some of which have already reported interesting results in early-phase clinical trials (70).

Table 1. Phase II and III randomized clinical trials evaluating HER2-directed treatments in gastric cancer.

Study	Design	Treatment Arm (A)	Treatment Arm (B)	Setting
ToGA (53)	Open-label, international, phase III, randomized	Trastuzumab + CDDP + 5FU/Cape	+ 5FU/Cap	First-line
LOGiC (66)	Double-blind, international, phase III, randomized	Lapatinib + CapeOX	Placebo + CapeOX	First-line
TyTAN (67)	Two-part, Asian, parallel-group, phase III	Lapatinib + paclitaxel	Paclitaxel	Second-line
GATSBY (68)	Open-label, international, phase II/III, randomized	T-DM1	Taxane	Second-line
JACOB (69)	Double-blind, international, phase III, randomized	Pertuzumab + Trastuzumab + CDDP + 5FU/Cape	Placebo + Trastuzumab + CDDP+ 5FU/Cape	First-line

WJOG7112G (71)	Open-label, Japanese, phase II, randomized	Trastuzumab + Paclitaxel	Paclitaxel	Second-line
DESTINY-Gastric01 (72)	Open-label, Asian, phase II, randomized	Trastuzumab deruxtecan	Paclitaxel or Irinotecan	Third-line and later-lines

Abbreviations: 5FU: 5-fluorouracil; Cape: capecitabine; CapeOX: capecitabine plus oxaliplatin; CDDP: cisplatin; ref, reference; T-DM1: trastuzumab emtansine.

Trastuzumab, the ToGA Trial and Resistance Mechanisms. Trastuzumab is a monoclonal antibody (mAb) that binds to domain IV of the extracellular segment of the HER2 receptor. Proposed mechanisms of Trastuzumab actions include (i) inhibition of ligand-independent homo-dimerization, (ii) antibody-dependent cellular cytotoxicity, (iii) inhibition of HER2-mediated signaling in cancer cells, and (iv) inhibition of tumor angiogenesis (73).

In 2010, the phase 3 ToGA trial first demonstrated the benefit of adding Trastuzumab to first-line chemotherapy in patients with HER2-positive (IHC 3+ or FISH amplified HER2/CEP17 ≥ 2) locally advanced, recurrent, or metastatic gastric or GEJ adenocarcinoma (53). Five hundred ninety-four patients were randomized to either trastuzumab combined with chemotherapy (fluorouracil or capecitabine plus cisplatin) or chemotherapy alone. The study met its primary endpoint of significantly improving median overall survival (OS) with Trastuzumab plus chemotherapy vs chemotherapy alone in the intent-to-treat population (13.8 vs 11.1 months, HR, 0.74; P = 0.0046). In a post hoc subgroup analysis, the OS benefit of adding Trastuzumab appeared limited to patients whose tumors were HER2 IHC 2+ and FISH positive or IHC 3+ (n = 446, 16.0 vs 11.8 months, HR, 0.65; 95% CI 0.51–0.83), but not in cases where tumors were IHC 0 or 1+ despite being FISH positive (n = 131, 10 vs 8.7 months, HR = 1.07).

Unfortunately, Trastuzumab resistance represents a major obstacle in HER2-positive patients as it almost inevitably occurs (74). Despite some mechanisms being shared between breast cancer and GC (e.g., the activation of downstream pathways, low levels of HER2 expression, etc.), several studies have suggested that there are other mechanisms of resistance that are specifically involved in GC (75). Among these, a number of reports have highlighted that HER2

expression may be lost in GC patients following disease progression on Trastuzumab (76) (77). In addition, unlike breast cancer, several aberrations are able to drive Trastuzumab resistance in GC, including mutations in EGFR, MET, KRAS, PI3K, and PTEN genes, as well as EGFR, MET, and KRAS amplifications (78).

The development and introduction of immune checkpoint inhibitors (ICIs) in GC has spurred a growing interest in combination strategies including ICIs and HER2-targeted treatments (79).

In fact, preclinical studies have suggested a synergistic activity for the combinations including trastuzumab and PD-1 inhibitors, resulting in T-cell activation and enhancing Antibody Dependent Cellular Phagocytosis (ADCC) in murine models (80). In a recent phase II trial, HER2-positive treatment-naïve patients receiving Pembrolizumab, Trastuzumab, platinum and fluoropyrimidine reported interesting results, with a 91% of response rate and median overall survival (OS) of 27.3 months (81). Ongoing clinical trials will probably shed further light on the role of this combination strategy, with the highly awaited results of a phase III study of Trastuzumab-chemotherapy plus Pembrolizumab versus Trastuzumab-chemotherapy plus placebo in the front-line setting (NCT03615326).

Trastuzumab deruxtecan T-DXd (DS8201). T-DXd is a novel HER2-targeting ADC composed of a Trastuzumab, an enzymatically cleavable peptide-linker, and a topoisomerase I inhibitor. After binding to HER2 on tumor cells, T-DXd is internalized, and the linker is cleaved within the tumor cells by lysosomal enzymes. Once released, the DX-8951 derivative (DXd) (which has more potent efficacy than irinotecan as a topoisomerase I inhibitor against various tumor xenograft models) binds to and inhibits topoisomerase I-DNA complexes, leading to the inhibition of DNA replication, cell cycle arrest, and tumor cell apoptosis. T-DXd has a higher drug-to-antibody ratio with homogenous conjugation compared with T-DM1. The high stability of the linker-payload of T-DXd in the plasma was demonstrated in vitro and in vivo, and the short half-life of DXd in the systemic circulation was also shown in vivo (82).

Notably, it has a bystander killing effect in vitro and in mouse xenograft models. In the presence of neighboring HER2-positive cells, adjacent HER2-negative tumor cells were also killed by T-DXd.

DESTINY-Gastric01 was a randomized phase 2 trial that evaluated T-DXd versus chemotherapy in a refractory population of patients with HER2-positive gastric and gastroesophageal adenocarcinoma who had progressed on ≥2 prior therapies, including Trastuzumab. T-DXd showed improvements in OS (12.5 vs 8.4 months) and response rate (RR) (51% vs 14%) compared with chemotherapy (83).

On 15 January 2021, the United States (U.S.) Food and Drug Administration (FDA) approved T-DXd for adult patients with metastatic GC who have received a prior Trastuzumab-based regimen, with the agent representing the second HER2 targeted treatment approved for HER2-positive GC. T-DXd had previously been approved in Japan on 25 September 2020 for the same indications. Moreover, the efficacy and safety of T-DXd is also under evaluation in an ongoing phase II, open-label, single-arm trial in Western countries, which is currently enrolling previously treated patients with advanced GC or GEJ cancers (NCT04014075). In contrast to the DESTINY-Gastric01, the study requires the central confirmation of the HER2 status on new tissue sample; lastly, the trial has ORR as the primary endpoint, based on an independent central review, and PFS and OS as secondary endpoints.

Novel anti-HER2 agents and strategies under investigation.

As summarized in Figure 11, HER2-targeted strategies in gastric cancer consist of monoclonal antibodies, TKIs, bispecific antibodies, antibody conjugates, and cellular- based therapies using T cells and NK cells. Currently, there are more than 30 ongoing clinical trials testing anti-HER2 therapy in gastric cancer which may inform the treatment landscape in options beyond Trastuzumab.

Margetuximab. Margetuximab is an Fc (fragment crystallizable region) engineered HER2-directed monoclonal antibody with enhanced ADCC activity (84); notably enough, promising antitumor activity has been reported in early-phase clinical trials on HER2-positive cancer patients, including low HER2-expressing GC (85). In particular, the combination of the PD-1 inhibitor Pembrolizumab plus Margetuximab has shown promising levels of activity in a phase Ib-II trial in HER2-positive GCs and GEJ cancers, with an ORR of 35.7% and a DCR of 67.9% in the

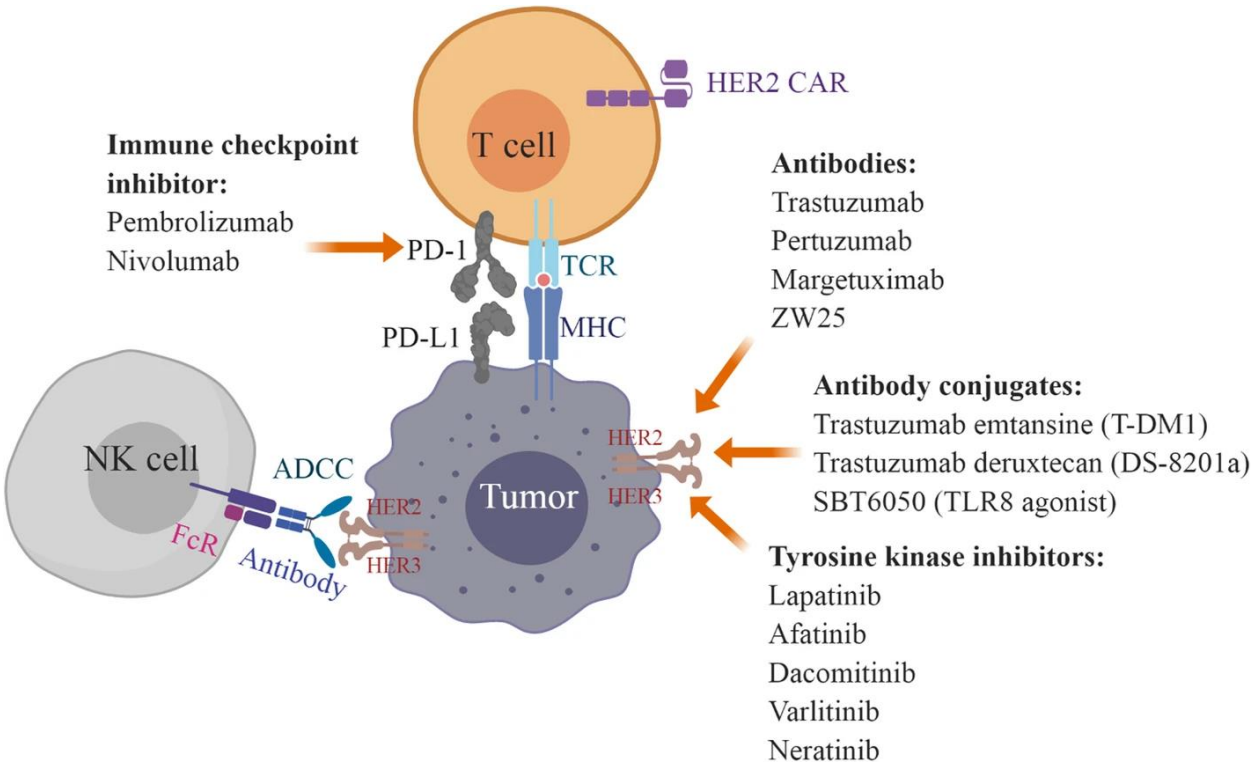


Figure 11: Strategies for targeting HER2-positive gastric cancer.

Anti-HER2 antibodies include Trastuzumab, Pertuzumab, Margetuximab, and ZW25.

Anti-Her2 antibody conjugates include Trastuzumab emtansine (T-DM1), Trastuzumab deruxtecan (DS-8201a), and SBT6050 (TLR8 agonist).

Tyrosine kinase inhibitors targeting HER2 include Lapatinib, Afatinib, Dacomitinib, Varlitinib, and Neratinib.

Fc receptors (FcR) expressed on NK cell(natural killer cell) bind to antibodies against HER2 and trigger anti-tumor immune response via antibody-dependent cellular cytotoxicity (ADCC). NK cell products in combination with Trastuzumab for HER2-positive tumors were under investigation.

Immune checkpoint inhibitors target program death 1 (PD-1)/programmed death-ligand 1 (PD-L1), the co-inhibitory signals for T cell antigen receptor (TCR) signaling, to enhance T cell anti-tumor immunity.

Chimeric antigen receptor (CAR)-T cells expressing HER2-specific CAR maybe an option for HER2-positive gastric cancer. Adapted from "Strategies for targeting HER2-positive gastric cancer" (86).

HER2 3+ and PD-L1 positive patient population (87). Based on this biological rationale and this preliminary evidence, the combination of the PD-1 inhibitor plus Margetuximab seems to show a synergistic activity, with a favorable safety profile. This preliminary evidence has supported further research in the same direction, as witnessed by the ongoing MAHOGANY randomized,

open-label phase II/III trial, comprising different cohorts and parts. In fact, the first part of cohort A MAHOGANY aims at assessing the ORR and the tolerability of the combination therapy of Margetuximab plus the PD-1 inhibitor Retifanlimab (MGA012) in HER2-positive (IHC 3+ or IHC 2+/FISH-positive) GCs (85); conversely, in cohort B patients with HER2-positive GC, regardless of the PD-L1 status, are randomized to receive (1) combination of chemotherapy plus Trastuzumab, (2) chemotherapy plus Margetuximab, (3) chemotherapy plus Margetuximab plus Retifanlimab, or (4) the PD-1 and LAG-3 inhibitor Tebotelimab (85).

ZW25. Another molecule under evaluation in this setting is the HER2-targeted bispecific antibody ZW25 which binds to two distinct HER2 epitopes (88). In preclinical studies, ZW25 showed promising activity in HER2-positive cancer cells lines and in breast, gastric, and ovarian CDX (Cell line-Derived Xenograft) and PDX (Patient-Derived Xenografts) models (89). In a phase I study evaluating ZW25 in HER2-positive solid tumors, four out of nine GC/GEJ cancer patients achieved partial response (PR) (90).

Besides the previously discussed agents, there are also some tyrosine kinase inhibitors (TKIs) under assessment in HER2-positive disease. Among these, the second-generation TKI **Tucatinib** has been suggested to be more potent than lapatinib with also a more favorable safety profile (91). Previous clinical trials evaluating Tucatinib in breast cancer have reported impressive results, especially in pretreated patients with brain metastases (92); moreover, this molecule has the potential to represent a promising therapeutic option when combined with other anticancer agents, given the encouraging responses observed in other malignancies, including colorectal cancer (93). Among the number of HER2-directed agents, there is a growing attention towards **MT-5111**, an immunotoxin whose action is based on the use of a Shiga-like toxin to enter into HER2-positive cells (94); notably enough, preclinical reports have suggested that MT-5111 could be combined safely with Trastuzumab, and MT-5111 is being investigated in breast cancer and GC patients (95).

4. OTHER RELEVANT THERAPEUTIC MOLECULAR TARGETS

Targeting angiogenesis pathways

Angiogenesis is necessary for tumors to grow beyond a certain size, survive or spread. Vascular endothelial growth factor (VEGF) and its receptors (VEGFR1, VEGFR2 and VEGFR3) are important players in the development of this process. Binding of the ligand VEGF-A to VEGFR-2 triggers a signaling cascade leading to endothelial cell proliferation, migration, new vessel formation, and sustained angiogenesis (96). Therefore, inhibition of the VEGF signaling has become a useful clinical maneuver in the treatment of several types of cancer.

Ramucirumab is a novel humanized IgG1 mAb that selectively binds to the extracellular ligand binding domain of VEGFR-2 and blocks VEGF-induced angiogenic signaling (97). In theory, this has the advantage of blocking signaling from VEGF isoforms other than VEGF-A. Its efficacy and safety in advanced GC were evaluated in two international, phase III, randomized, double-blinded and placebo-controlled studies. In the REGARD trial, a total 355 advanced gastric or GEJ cancer patients progressing after first-line platinum or fluoropyrimidine-based combination chemotherapy were randomized to receive best supportive care (BSC) plus either Ramucirumab or placebo (13). Patients receiving Ramucirumab had a significantly improved median OS and PFS than patients receiving placebo. After presentation of these results, Ramucirumab was approved for the second-line therapy of advanced GC. The RAINBOW study tested Ramucirumab in combination with paclitaxel in metastatic GEJ or gastric adenocarcinoma patients who experienced disease progression after first-line platinum- and fluoropyrimidine-based chemotherapy (14). In this study, 665 patients were randomly assigned to receive Ramucirumab or placebo plus paclitaxel. The study showed that an effective second-line treatment may improve the duration of survival in metastatic GC, and it is the only study to date to demonstrate a 2-months improvement in OS

in this setting. Therefore, Ramucirumab is the first antiangiogenic agent to demonstrate activity for advanced GC, and now approved both as monotherapy and in combination with paclitaxel for this malignancy.

Targeting programmed cell death 1.

Mismatch repair (MMR) genes are responsible for fixing errors that occur during deoxyribonucleic acid (DNA) replication. Tumors with defects in the mismatch repair system (MMR-deficient [dMMR]) harbor significantly more mutations than tumors with intact MMR machinery (MMR- proficient). dMMR tumors are vulnerable to mutations in microsatellites, which are repetitive sequences of nucleotide bases found throughout the genome, leading to high levels of MSI. Across tumor types, patients with dMMR cancers are more likely to respond to PD-1 blockade than those with MMR-proficient cancers (98). In part, this is because of high levels of neoantigens and PD-L1–positive T-cell infiltration in dMMR tumors.

Pembrolizumab is a humanized monoclonal antibody that inhibits PD-1/PD-L1 axis by binding to PD-1 receptors on T cells, thereby blocking PD-1 ligands (PD-L1 and PD-L2) from binding. PD-1 blockade results in removal of the physiologic brake on an active immune system and induces antitumor response. KEYNOTE-158 was a phase 2 trial that enrolled patients with treatment-refractory, noncolorectal MSI-H/dMMR cancers to receive pembrolizumab (99). Of the 24 patients with gastric cancer, there were 11 responses (including 4 complete responses), and the median PFS was 11 months. This trial ultimately led to the tissue-agnostic US Food and Drug Administration (FDA) approval of pembrolizumab for patients with unresectable or metastatic MSI-H or dMMR tumors of any solid tumor type, including gastric cancer, who progressed after prior treatment and have no satisfactory alternative treatment (100).

KEYNOTE-059 was a phase 2 trial of pembrolizumab therapy in patients with advanced gastric cancer who had disease progression after ≥2 lines of therapy (101). Overall, the objective response rate (ORR) was 11.6%, and the median duration of response (DoR) was 8.4 months. However, in PD-L1–positive (CPS ≥1) patients, the ORR was 15.5%, and the median DoR was 16.3 months. These results were the basis of the FDA approval of pembrolizumab for third-line treatment of PD-L1–positive (CPS ≥1) gastric adenocarcinoma.

On May 5, 2021, the Food and Drug Administration granted accelerated approval to pembrolizumab in combination with Trastuzumab, fluoropyrimidine- and platinum-containing chemotherapy for the first-line treatment of patients with locally advanced unresectable or metastatic HER2 positive gastric or gastroesophageal junction (GEJ) adenocarcinoma.

Approval was based on the prespecified interim analysis of the first 264 patients of the ongoing KEYNOTE-811 trial, a multicenter, randomized, double-blind, placebo-controlled trial in patients with HER2-positive advanced gastric or gastroesophageal junction (GEJ) adenocarcinoma who had not previously received systemic therapy for metastatic disease. (102)

5. TRANSLATIONAL CANCER RESEARCH

Objectives of translational cancer research range from the biologic understanding of the disease to the development of new treatments; hence preclinical models are required to investigate biological mechanisms and features. Regarding drug development and testing, several models have been proposed and exploited through the years, such as murine models and cancer cell lines. Numerous studies were led to establish basic methodology and a systematic approach for preclinical testing of anticancer molecules both *in vitro* and *in vivo* (103) (104).

The majority of current knowledge about the biology of cancer cells has been obtained using cell cultures that allowed to discover gene alterations in cancer, identify aberrant signaling pathways, and screen new chemical entities as potential chemotherapeutic agents. However, cell lines have important limitations (105). Lack of predictive value is one of the most relevant problem: this is maybe due to gain and loss of genetic information, alteration of growth and acquisition of invasive properties, as a consequence of the process of generating cancer cell lines themselves and of selection (106). Moreover, cell lines are representative of an only subset of tumors, missing the ability to preserve neoplastic heterogeneity (107) (144). Furthermore, studies performed in cell lines miss the regulatory role of tumor microenvironment. For these and other reasons, 3D tissue culture and organoid systems were developed.

Organoids are an advancement of traditional tissue culture that is meant to mimic more closely the 3D architecture of primary tumors. Hans Clevers defined an organoid as "a 3D structure grown from stem cells and consisting of organ-specific cell types that self-organize through cell sorting and spatially restricted lineage commitment" (108). They self-organize and mimic the general architecture of the tissue of origin, and, importantly, maintain these characteristics over successive passages. This relevant *in vitro* model offers advantages for studying tumor progression, treatment responsiveness, and interactions with the immune system and the tumor microenvironment. However, a central question to consider with organoids, given the dynamic and heterogeneous nature of the cancer genome, is how well do organoids reflect the

genetic and mutational profile of the parent tumor and how stable is the genetic over the study/treatment period? In other words, do the conditioned and semi-artificial culture conditions of organoid growth environments result in deviation of tumors from their inherent genetic mutational evolution?

To overcome these critical issues other preclinical cancer models have been introduced. Patient-derived tumor xenografts are one of these emerging new tools, attempting to improve the drug development process (Figure 12) (109).

5.1 PATIENT-DERIVED CANCER XENOGRAFT MODELS (PDX)

Patient-derived cancer xenografts are originated by implanting patients' tumors as pieces or single-cell suspensions on the dorsal region of immunodeficient mice (subcutaneous implantation) or in the organ in which the original tumor developed (orthotopic implantation). Tumors to be implanted are collected by surgery or biopsy procedures (110) (111). Metastases implantation may be an option and several studies show that this type of lesions has a higher engraftment rate (112). NOD/ SCID or NOD/SCID/IL2λ-receptor null mice are the mouse models with the highest engraftment rates because of their severe immunosuppression (113).

As described above, two methods of implantation are possible: subcutaneous and orthotopic. The former is more frequently exploited, because of the lower complexity of the implantation technique. On the other hand, orthotopic tumor implantation provides a translational advantage, mainly because the tumor develops in its original anatomic microenvironment and allows metastasis development. However, this technique is more labor-intensive and expensive, often requiring complex surgery and imaging methods to monitor tumor growth (114).

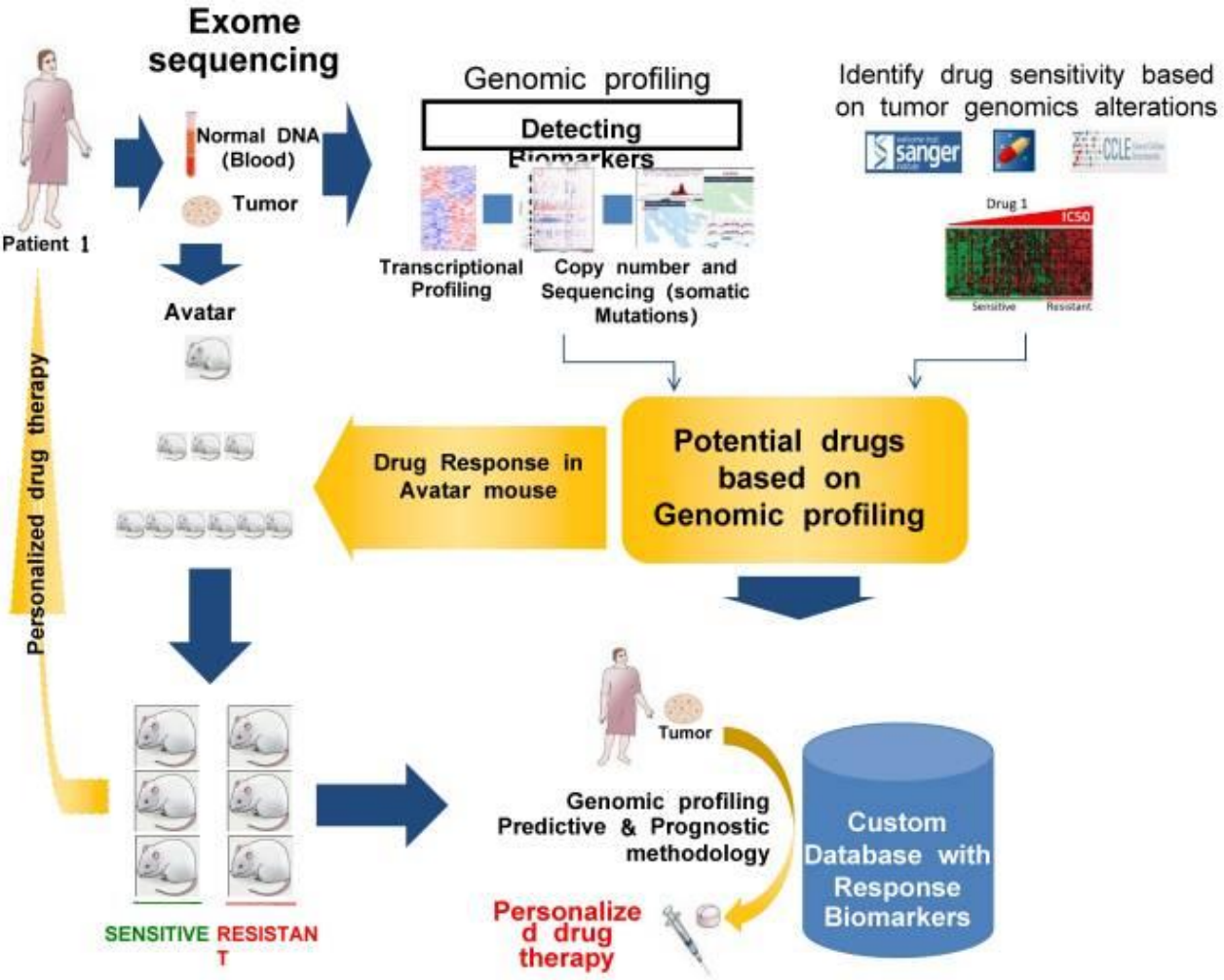


Figure 12: Personalized medicine strategy. This figure shows a strategy for individualized medicine that integrates genomic analysis of a patient tumor with testing in PDX models. Molecular analysis of a patient tumor can lead to the identification of potential therapeutically targetable alterations. Mining of genomic–drug response databases could result in several potential therapeutic regimens for a given patient. PDXs can be exploited to test and rank these potential treatments to be administered to the patient. Adapted from "Patient-derived xenograft models: an emerging platform for translational cancer research" (109).

5.2 SALIENT FEATURES OF PDX MODELS

The importance of PDX models is related to their ability to retain the salient characteristics of the donor tumors, including tissue structure and molecular features. This is an extremely important point because PDXs, for this reason, may represent enhanced preclinical tools, being predictive of human cancer biology and patient response to treatments. Numerous studies which compared PDXs, and donor tumors demonstrated that PDX models show the same fine tissue structure and subtle microscopic details, such as gland architecture, mucin production, or cyst development of the original specimen (115). Moreover, gene expression profiling analysis of donor tumors and the corresponding PDXs are mostly overlapping, except for genes involved in the stromal compartment and immune function, because the human stroma is replaced by murine elements. Molecular features also show extraordinary concordance between PDX and donor tumor: studies evaluating copy-number alterations and exome sequencing report even a higher frequency of genomic alterations in the PDX model, due to an increased purity of the human tumor DNA which is not contaminated by normal DNA from the human stromal tissue (116). Furthermore, the functional features of the grafted tumor remain almost the same during mouse-to-mouse propagation; indeed, drug treatments of PDX models from different passages show similar effects across generations (117) (118). Importantly, several studies highlighted the similarity between the activity of drugs in PDX models and in the corresponding clinical trials (109); in addition, emerging studies in which human patients have been treated with drugs previously tested in their PDX counterparts demonstrated a high predictive power. For these reasons, PDXs may be considered models effectively correlating with clinical outcome (119).

5.3 LIMITATIONS OF PDXs

Recently, groups with wide expertise have sought to assess the validity of PDX models in recapitulating different types of cancers and have highlighted many of the limitations and challenges of PDX models (120) (121) (122). All groups working with PDXs models argue that more studies should incorporate standardized validation tools to improve the reproducibility and to increase success rates of translational studies (123) (109) (124) (122).

Among some of the main challenges is the gradual replacement of human stroma with mouse stroma. In fact, after few passages, tumor-associated stroma is replaced with murine-derived ECM (extracellular matrix) and fibroblasts, causing changes in the paracrine regulation of the tumor that might interfere with drug distribution and effectiveness (125).

Another challenge is the route of implantation. There are open questions surrounding the most favorable route of administration indicating the need for validation studies to address the optimal implantation site. Although orthotopic models seem to better mimic metastatic cancer models, sub- cutaneous administration is more commonly used because it is easier to assess drug efficacy (126).

The time course of engraftment is also a formidable challenge and is a limiting factor in the use of PDX models for co- clinical trials. Some models take 4–8 months to establish and this is more than what patients can wait to start treatment. In order to overcome this issue, some groups are switching to the use of organoid models to evaluate potential treatment sensitivity (108) (127).

Lastly, in order to establish standard PDX models, a key requirement is that the mice cannot have an intact immune system. This has impeded the establishment of PDXs mice in studies assessing immune checkpoint–blocking agents (128) (129). This is also driven, in part, by gradual replacement of engrafted stromal cells (and immune cells found in the tumor) with mouse cells leading to a more murine-like tumor micro-environment (121). For these reasons, the development of humanized PDX models where the immune system is reconstituted in the PDX-implanted mouse represents a potential advancement for researchers (129) (130). Nevertheless, despite these challenges, PDX models are considered among the most robust and clinically relevant models for drug screening and drug discovery.

6. EXTRACELLULAR VESCICLES (EVs)

Cell to cell communication is pivotal for all multicellular organisms. Cells exchange information through the secretion of soluble factors or direct interaction. In addition, most eukaryotic cells release membrane-derived vesicles that can have an impact on both neighboring and distant cells (131). Such extracellular vesicles (EVs) were initially described nearly 30 years ago when two independent groups observed that multivesicular bodies in reticulocytes release such vesicles into the extracellular space (132). Since then, extracellular vesicles have been purified from nearly all mammalian cell types, including stem cells, cells of the immune and nervous systems as well as numerous cancer cell lines (133).

Perhaps unsurprisingly, extracellular vesicles were initially regarded as membrane debris with no real biological significance. However, in 1996, Raposo et al. showed that extracellular vesicles could stimulate adaptive immune responses (134).Since then, the importance of extracellular vesicles in intercellular communication, via the transfer of proteins, lipids and nucleic acids, has been reported in numerous studies (135) (136). Extracellular vesicles have been isolated from most body fluids and it is increasingly evident that they have a key role not only in the regulation of normal physiological processes, such as stem cell maintenance, tissue repair, immune surveillance, and blood coagulation, but also in the pathology underlying several diseases (137) (138) (139).

On the basis of the mechanisms of biogenesis, at least two classes of EVs have been identified. Ectosomes (microvesicles (MVs) and oncosomes) are formed by direct plasma membrane budding and range from 100 to 1,000 nm in diameter (140). The size and biogenesis of ectosomes is a distinguishing feature for a smaller subset of EVs, known as exosomes. (140). Exosomes are nano-sized vesicles (30–150 nm) originating from the endocytic pathway. Exosome biogenesis begins with invagination of the endosomal limiting membrane and formation of intraluminal vesicles (ILVs), resulting in a specialized cell compartment, known as multivesicular body/endosome (MVB/MVE). Extracellular release of ILVs is coordinated by the

fusion of MVBs with the plasma membrane, resulting in the release of ILVs as exosomes (Figure 13).

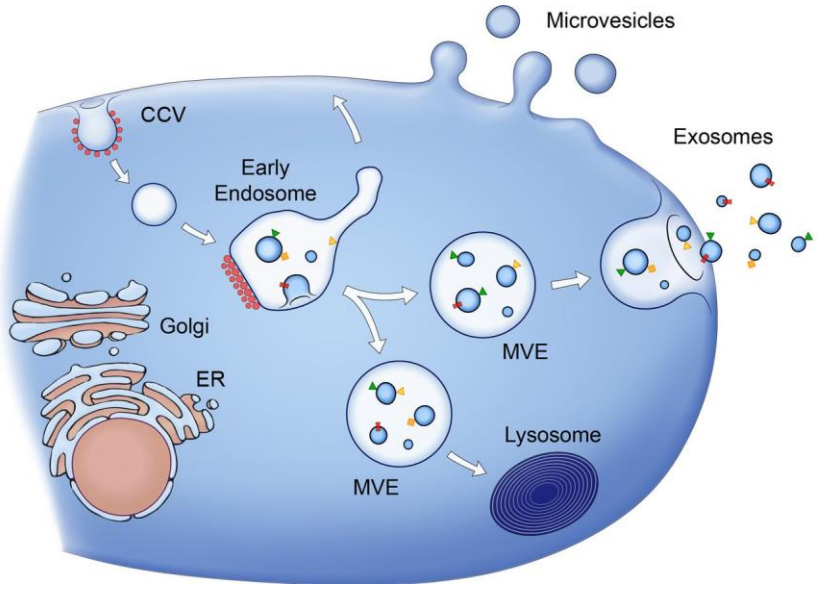


Figure 13: Release of microvesicles (MVs) and exosomes. MVs bud directly from the plasma membrane, whereas exosomes are represented by small vesicles of different sizes that are formed as the ILV by budding into early endosomes and MVEs and are released by fusion of multivesicular endosomes (MVEs) with the plasma membrane. Other MVEs fuse with lysosomes. The point of divergence between these types of MVEs is drawn at early endosomes, but the existence of distinct early endosomes feeding into these two pathways cannot be excluded. Red spots symbolize clathrin associated with vesicles at the plasma membrane (clathrin-coated vesicles (CCV)) or bilayered clathrin coats at endosomes. Membrane-associated and transmembrane proteins on vesicles are represented as triangles and rectangles, respectively. Arrows represent proposed directions of protein and lipid transport between organelles and between MVEs and the plasma membrane for exosome secretion. Adapted from "Extracellular vesicles: Exosomes, microvesicles, and friends" (141).

6.1 BIOLOGICAL ROLES OF EXTRACELLULAR VESICLES

Extracellular vesicles exert their effects on fundamental biological processes in a pleiotropic manner, directly activating cell surface receptors via protein and bioactive lipid ligands, merging their membrane contents into the recipient cell plasma membrane, and delivering effectors including transcription factors, oncogenes, small and large non-coding regulatory RNAs such as

microRNAs , mRNAs and infectious particles into recipient cells (137) (138) (139). In this way, extracellular vesicles participate in the maintenance of normal physiology. Extracellular vesicles can thus be regarded as signalosomes: multifunctional signaling complexes for controlling fundamental cellular and biological functions. For example, in the regulation of immune responses, depending on the status of particular immune cells, extracellular vesicles might trigger adaptive immune responses or suppress inflammation in a tolerogenic manner (136). Extracellular vesicles have been shown to confer immune suppression by several mechanisms: they can enhance the function of regulatory T cells, suppress natural killer (NK) and CD8+ cell activity and inhibit monocyte differentiation into DCs as well as DC maturation (142) (143) (144). By contrast, the effects of immune activation can be mediated by extracellular vesicle-promoted proliferation and survival of hematopoietic stem cell and the activation of monocytes and B cells (145) (146) (147). Extracellular vesicles have also been implicated in cell phenotype modulation for example, in shifting the bone marrow cell transcriptome and proteome towards a lung phenotype in vivo (148). Importantly, several reports have implicated extracellular vesicles in stem cell maintenance and plasticity, indicating that stem cell-derived extracellular vesicles have a pivotal role in tissue regeneration following injury (146).

6.2 EVS AND CANCER

Intercellular communication between cancer cells and the tumor microenvironment is a crucial process in cancer progression (149) (150). Historically, communication between cancer cells and surrounding stromal cells was thought to be governed primarily by direct cell- to-cell contact as well as secretion and uptake of soluble cytokines and growth factors (149) (150). However, it has become clear that a new paradigm of intercellular communication, involving information exchange via secreted vesicles, is an essential process in both normal and pathological processes (151) (152). Over the past decade, there has been a significant increase in knowledge of the role of extracellular vesicles (EVs) during cancer initiation and progression.

The role of EVs in cancer is diverse and they contribute to many of the hallmarks of cancer, including cell proliferation and migration, angiogenesis, evasion of cell death, and invasion and

metastasis (153). Cancer-derived as well as tumor microenvironment EVs are constitutively released; however, EV release is increased in response to common microenvironmental perturbations in pH, oxygen tension and inflammation, as well as the therapeutic stresses of chemotherapy or irradiation. Furthermore, in response to various stresses, the biomolecular properties and cargo of EVs are altered, leading to diverse signaling mechanisms involved in promoting cancer progression. (154) (155) (156) (157).

The understanding of EV biology has matured to the point where it is known that the diverse biomolecular constituents of EVs are reflective of their cell of origin (158) (159). Moreover, it is starting to unravel how oncogenic perturbations are involved in EV biogenesis and specific cargo packaging in cancer cells (160). This is highly pertinent as understanding the role of oncogenic alteration in EV biology will uncover therapeutically targetable, tumor-specific EV pathways and content, which could possibly generate novel classes of clinical biomarker assays and cancer therapies. Overexpression of various oncogenes alters the biomolecular cargo of EVs and MVs through inclusion of oncogenic molecules, such as KRAS and EGFR variant III in colon cancer and glioblastoma, respectively (161) (162), and/or by alteration of the global protein and/or nucleic acid content in EVs. Proteomic analysis of EVs from a panel of 60 cancer cell lines from the National Cancer Institute (NCI-60) demonstrated that only a small subset of EV cargo proteins is shared among the nine different cancer types in the NCI-60 panel. Most of more than 6,000 proteins were unique for each cancer type, indicating that each cancer cell type secretes EVs with a unique proteomic cargo (163). That is, the enrichment of specific proteins in EVs derived from the same cancer type indicates that protein cargo could potentially be used for the identification of the tissue from which an EV originates (163).

Moreover, due to their presence and stability in most bodily fluids and resemblance of their contents to parental cells, EVs have a great potential to serve as a liquid biopsy tool for various diseases (164) (165). In particular, cancer derived extracellular vesicles likely serves as biomarker for early detection of cancer as they carry the cargo reflective of genetic or signaling alterations in cancer cells of origin. Exosome based liquid biopsy merits consideration over conventional tissue biopsy for following reasons. It provides the convenient and non-invasive way of diagnosis over tissue biopsy that requires surgery. The small sample size of tissue biopsy cannot provide

the detailed information of genetic heterogeneity within the primary tumor or metastasized secondary tumors. However, exosomes shed from heterogeneous cancers can be collected at once and provide the dynamic information from the tumors at the time of blood drawing.

6.1 THE ROLE OF EVs IN GASTRIC CANCER

Emerging evidence indicates that extracellular vesicles are critically involved in GC progression including tumorigenesis, metastasis, angiogenesis, immune evasion, and drug resistance (Figure 14). Qu et al. first described the role of EVs in GC in 2009. They reported that GC cell derived EVs promoted GC cell proliferation by activating PI3K/Akt and MAPK/ERK pathways (166). The pre-exposure of GC cells to their derived EVs resulted in enhanced tumor growth and angiogenesis in the NOD/SCID mouse model, suggesting a protumorigenic role of extracellular vesicles as macro-messenger by delivering signals and molecular cargos (167). Additionally, Li and colleagues found that EVs from gastric cancer cells significantly increased gastric cancer cell proliferation and invasion and that they could mediate GC metastasis to local or distant tissues and organs (168). Tanaka and colleagues demonstrated that the incorporation of GC-derived EVs induced peritoneal mesothelial cell (PMC) infiltration, which in turn accelerated tumor invasion in the gastric wall, and PMC-led cancer cell invasion in disseminated tumors within the abdominal wall and diaphragm (169). This hypothesis was subsequently strengthened by Deng, with the description that GC cells derived exosomes induced injury of PMCs through apoptosis and mesothelial-to-mesenchymal transition (MMT), resulting in mesothelial barrier destruction and peritoneal fibrosis (170). These findings support that EVs play a crucial role in remodeling the premetastatic microenvironment and mediating peritoneal metastasis. Another important feature of GC-derived EVs is their ability to modulate tumor immunity. Wang et al. reported that exosomes derived from gastric cancer cells can induce the production of PD-1^{+} tumor-associated macrophages (TAMs), which interact directly with PD-L1^{+} cells to produce IL-10, resulting in dysfunction of CD8+ T cells and favorable conditions for GC progression (171). Zhang et al. demonstrated that GC cell-derived exosomes could induce neutrophils to polarize to N2 tumor-associated neutrophils (TAN), thus promoting gastric cancer cell migration (172).

Extracellular vesicles can also mediate drug resistance in GC. Ji et al. reported that mesenchymal stem cells (MSCs) derived exosomes could induce the resistance of GC cells to 5-fluorouracil (5-FU), which may be associated with their role in activating Ca2+/Raf/MEK/ERK signaling pathway and upregulating the expression of multidrug resistant proteins in GC cells (173).

Moreover, the unique expression pattern and the relative stability of its contents have made extracellular vesicles a new candidate for tumor liquid biopsy: increasing studies have shown that EVs may have a great potential to serve as biomarkers for the early diagnosis, the prediction of prognosis, and the evaluation of therapy effect in GC (174) (175).

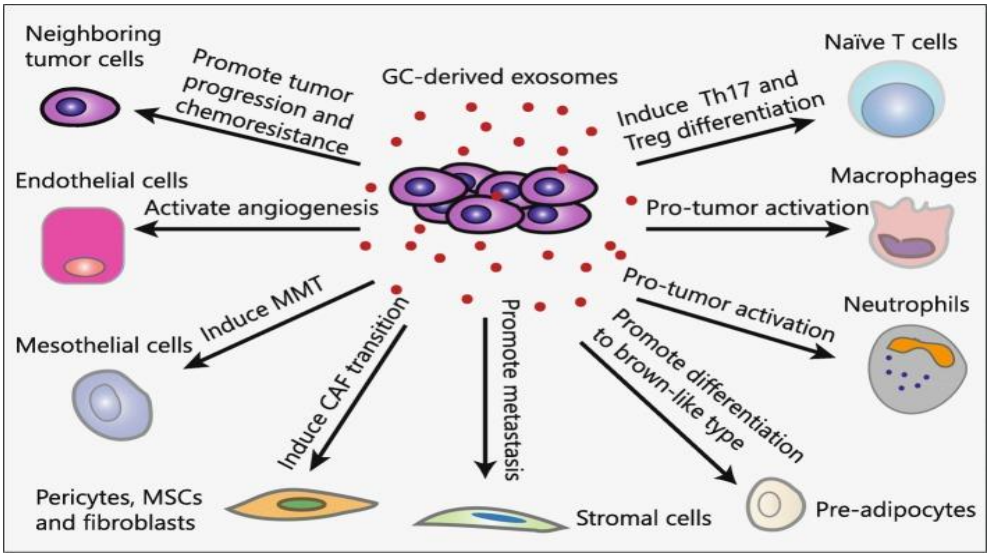


Figure 14: Roles of tumor cell-derived EVs in GC. EVs are critically involved in GC progression including tumorigenesis, metastasis, angiogenesis, immune evasion, and drug resistance by transferring functional biomolecules. GC cell-derived extracellular vesicles can modulate immunity by activating pro-tumor phenotypes of neutrophils and macrophages and inducing the differentiation of T cells to Th17 and Treg cells. GC cells derived EVs can convert pericytes, fibroblasts and MSCs into myofibroblasts to facilitate tumor angiogenesis and metastasis. Moreover, GC cell-derived EVs can activate endothelial cells to support tumor angiogenesis and promote significant adhesion between mesothelial and GC cells. GC cell-derived EVs help to create a favorable microenvironment for liver metastasis by acting on liver stromal cells. In addition, pre-adipocytes prefer to differentiate into brown-like type by GC cells derived EVs. Adapted from "Exosomes in gastric cancer: Roles, mechanisms, and applications" (176).

MATERIAL AND METHODS

PATIENTS AND TUMOR SAMPLES

Tumor samples (from gastric and gastroesophageal junction carcinomas) and matched normal samples were obtained from patients undergoing surgery in 15 Italian Hospitals.

All patients provided written informed consent; samples were collected, and the study was conducted under the approval of the Review Boards of all the Institutions. The study was done in accordance with the principles of the Declaration of Helsinki, the International Conference on Harmonization and Good Clinical Practice guidelines and GDPR (General Data Protection Regulation). Clinical and pathologic data were entered and maintained in our prospective database. All the samples havebeen anonymized before being shipped to the Candiolo Cancer Institute (Candiolo, Torino, Italy). No reference to the patients can be inferred from the histological and molecular characterization presented in the work.

ANIMALS

Guidelines for Care and Use of Laboratory Animals were followed during the investigation. Ethical Commission of the IRCC in Candiolo and the Italian Ministry of Health approved all animal procedures. NOD SCID mice were purchased by Charles River (Milan, Italy).

XENOGRAFT TRANSPLANTATION EXPERIMENTS

Tumor material not required for histopathologic analysis was collected and placed in medium 199 supplemented with 100 µg/mL levofloxacin. The surgical sample was cut into 25- to 30-mm3 pieces in antibiotic-containing medium; some of the pieces were incubated overnight in RNAlater (Invitrogen, California, USA) and then frozen at –80°C for molecular analyses; 2 other pieces were coated in Matrigel (BD Biosciences, Franklin Lakes, New Jersey, USA) and subcutaneously implanted in 2 different 4- to 6-week-old female NOD (nonobese diabetic)/SCID (severe combined immunodeficient) mice. After mass formation, the tumors were analyzed for genetic identity with the original tumor (by short tandem repeat profiling, Cell ID) and for maintenance of HER2 amplification; tumors were then passaged and expanded until production of a cohort of mice bearing homogeneous tumors. To obtain such uniform cohort we transplanted twice as many animals. Established and randomized tumors (average volume 250 mm^3) were treated for the indicated days with the following

regimens (either single agent or combination): vehicle (saline) per os; Trastuzumab 30 mg/ kg, weekly ip; Pertuzumab 20 mg/kg, weekly ip; Lapatinib 100 mg/kg, daily, per os; TDM1 10 mg/kg, weekly iv, DS-8201a 10 mg/kg, weekly iv; crizotinib 25 mg/kg, daily, per os. Tumor size was evaluated once weekly by caliper measurements and approximate volume of the mass was calculated using the formula $4/3\pi(D/2)(d/2)2$, where D is the major tumor axis and d is the minor tumor axis.

RESPONSE TO TREATMENT

The response in mice has been evaluated using RECIST 1.1- like criteria (177), i.e. progressive disease (PD): ≥ 35% increase from baseline; partial response (PR): ≥ 50% reduction from baseline; stable disease (SD): intermediate variations from baseline. Statistical testing for pharmacological experiment was performed with GraphPad PRISM Soft- ware 8.0, using Two-way ANOVA followed by Bonferroni multiple comparisons correction. Statistical significance: ns = not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

PRIMARY CELL CULTURES

Cells were derived from the PDX tumor after collagenase 1 digestion (Sigma- Aldrich,St Louis, MO, USA). After 1 hour incubation at 37°C, suspension was collected and centrifuged at 800 rpm for 5 minutes. After several washing and centrifuging with Leibovitz's L- 15 (Sigma-Aldrich, St Louis, MO, USA) the pellet was suspended in medium to allow the 2D culture in coat plate with collagen. Cells were cultured in Iscove medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin.

Organoids were derived from the PDX tumor after collagenase 1 digestion (Sigma Aldrich, St Louis, MO, USA). After 20 minutes incubation at 37°C, suspension was collected and filtered through Cell strainer Falcon© (Corning Incorporated, Corning, NY, USA) 70 µm. Subsequently, suspension was centrifuged at 300 rpm for 5 minutes and the pellet was resuspended in 30/45 µl of Matrigel (BD Biosciences, Franklin Lakes, New Jersey, USA) to allow the 3D culture and to form organoids. Organoids were cultured in LWRN medium (a conditioned medium from the LWRN cell line) diluted with Advanced DMEM/F12 medium (Sigma-Aldrich, St Louis, MO, USA) (1:1) supplemented with 10% FBS, 100 units/mL penicillin.

CELL LINES

HEK293T,OE19,NCI-N87,OE33,MKN7,SKGT2,TE-4,TE-6 cells were obtained from ATCC. Cells were cultured in RPMI medium 1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. LWRN is a murine cell line stably producing factors essential for organoids growth (R-spondING1 and Wnt3a). This cell line was kindly given by Dr. Adam J. Bass (Institute for Systems Biology, Boston, MA, USA).

EBV EVALUATION

Detection and quantification of EBV DNA were performed using the EBV Q-PCR Alert KIT (ELI Tech Group S.p.A., Poteau, France). The real-time amplification assay was carried out on ABI 7300 Real-Time PCR System instrument (Applied Biosystems, USA). PDXs were classified: EBV high (with high EBV burden, > 1000, Equivalent EBV Genomes/reaction (gEq)), EBV intermediate (75–1000 gEq) or EBV low/neg (< 75 gEq). Tumors scored as EBV high, or intermediate were considered as EBV-positive.

WESTERN BLOT ANALYSIS

Cells were treated for 24 hours with the indicated drugs, used at the concentration corresponding to IC50 in viability assays (as reported in figure legends). Whole-protein extracts were prepared using Laemmli buffer and quantified using the BCA Protein Assay kit (Pierce, Rockford, IL, USA). Each protein sample was resolved on sodium dodecyl sulfate polyacrylamide gels (8%), transferred onto polyvinylidene difluoride membranes (Millipore Corporation, Billerica, MA, USA), blocked for 1 h at room temperature in 10% BSA in TBS-Tween, and incubated with the appropriate primary antibodies according to the manufacturer's instructions. anti-HER2 (sc-284), anti-EGFR (sc-03G) Alix (C-11) and CD9 (C-4) were from Santa Cruz Biotechnology (Dallas, Texas, USA), antibodies against phosphorylated HER2 (Tyr1248, #2247), phosphorylated MAPK (Thr202/Tyr204, #9101), phosphorylated AKT (Ser473, #4060), Phospho-S6 Ribosomal Protein (Ser235-6, #4858), total AKT (#9272), and MAPK (#9102) were from Cell Signaling Technology (Danvers, MA, USA). Antibody against phosphorylated EGFR (Tyr1068 #ab5644) was from Abcam (Cambridge, UK). Secondary antibodies were from

Amersham. Detection was performed with ECL system (Amersham).

DRUGS

Trastuzumab, Pertuzumab and T-DM1 were provided by Genentech (San Francisco, CA, USA) (MTA#OR-214409). DS8201 was provided by Daiichi Sankyo Company (Tokyo, JA). MM-121 was provided by Merrimack (Cambridge, MA, USA). Lapatinib was provided by the Hospital Pharmacy.

CELL VIABILITY ASSAY

For growth curve and cell viability assay cells were seeded in triplicates in 96-well culture plates coated with collagen. The drugs were added the day after. Media and drugs were changed once after three days. Trastuzumab, Pertuzumab, Lapatinib were used at the indicated concentrations.

Organoids were seeded in quadruplicates in 96-well culture plates. When organoids formed, drugs were added. Medium and drugs were changed once after three days. After 3 or 6 days of growth, cell viability of cells and organoids was measured by using Cell Titer- Glo Luminescent Cell Viability Assay (Promega), which directly measures the intracellular ATP content, resulting in quantification of the number of healthy cells in culture.

ANALYTE EXTRACTION

Genomic DNA was isolated using the Blood & Cell Culture DNA Midi Kit (Qiagen, Hilden, Germany). DNA concentrations were quantified using the Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

CNV EVALUATION BY REAL TIME qPCR

Quantitative PCR experiments for estimation of HER2 or KRAS copy number variations were performed in triplicates using 2ng total gDNA as a template, with the Human TaqMan Copy Number Assays ID Hs02876245_cn (HER2), ID Hs04942325_cn (EGFR), ID: Hs04993403_cn (MET), ID Hs01472955_cn (FGFR2) or ID Hs06936191 (KRAS), the TaqMan Copy Number

Reference Assay RNase P 4316831 and GREB1 Hs01738470_cn (Thermo Fisher Scientific)PCR runs were performed with ABI Prism 7900HT (Thermo Fisher Scientific).

MSI EVALUATION

MS status was evaluated with the MSI Analysis System version 1.2 kit (Promega). MSI analysis was performed according to the manufacturer's directions. The pathologist interpreted microsatellite instability at ≥2 mononucleotide loci as MSI, instability at a single mononucleotide locus and no instability at any of the loci tested as microsatellite stable (MSS).

GENOMIC SEQUENCING

DNAs extracted from PDX models along with a sample of normal germline DNA from each patient were collected for next generation sequencing. Using standard methods, Illumina sequencing libraries were generated and subjected to hybrid capture with a focused targeted bait set of 243 genes selected based on their alteration in prior studies of gastroesophageal cancer [19, 23]. GTR0455 has been sequenced for Whole Exome on Illumina NovaSeq platform using the Agilent SureSelectXT Human All Exon V6 library (Macro- gene Inc, Seoul, Korea).

HER2 FLUORESCENT IN SITU HYBRIDISATION (FISH) ANALYSIS

Dual color FISH analysis was performed using a commercial HER2 amplification probe (Dual-color FISH kit; GP Medical Co; Abbott Laboratories, Abbott Park, Illinois, USA). The paraffin sections were deparaffinized, air-dried, incubated in pretreatment solution at 98°C for 15 minutes in Heat Pretreatment Solution (Spot-light Tissue pretreatment kit, Life Technologies, CA, USA), and subsequently immersed in purified water. The slides were then treated with Enzyme Reagent (Spot-light Tissue pretreatment kit, Life Technologies, CA, USA) in a humidified box for 30 to 40 minutes at room temperature and washed in purified water. After air dehydration, 10 µL of probe mixture were applied to each sample. The slides were then coverslipped and sealed with rubber cement. Slides and probes were co- denatured at 73°C for 3 minutes and hybridized at 37°C for 16 hours in the dark (Top Brite, Resnova S.r.l., ROMA, ITALY). A post-hybridization wash was performed in 2×SSC–0.3% NP-40 at 73°C for

3 minutes. Finally, the slides were dehydrated, mounted with DAPI (4',6- diamidino-2-phenylindole; HER2 gene amplification was considered positive when its exhibited ratio of HER2:CEP17 (centromeric probe 17) was ≥ 2 in a minimum of 60 counted cancer cell nuclei, or when an HER2 Signal cluster was observed.

HER2 IMMUNOHISTOCHEMESTRY (IHC): HERCEPTEST ANALYSIS

Formalin-fixed, paraffin-embedded sections (4 µm thick) were mounted on silane-coated slides. Sections were dewaxed in xylene and rehydrated through graded alcohols. All sections were rinsed in deionized water. Immunostaining for HER2 protein was also performed using the HercepTest kit (Dako, Glostrup, Denmark) with strict adherence to the advised protocol. Antigen retrieval was achieved by immersing sections in a 1:10 dilution of epitope retrieval solution (Dako) at 95° to 99°C for 40 min. Sections were allowed to cool for 20 min at room temperature before rinsing in wash buffer. Sections were then incubated in peroxidase-blocking reagent for 5 min, rinsed with wash buffer, covered with 100 µl of primary antibody for 30 min and rinsed in wash buffer. Sections were then covered with 100 µl of visualization reagent (Dako), rinsed in wash buffer and then developed with diaminobenzidine tetrahydrochloride prior to counterstaining with hematoxylin. Finally, sections were dehydrated through graded alcohols and mounted in resinous mountant. Known HER2-expressing breast cell lines and breast and NSCLC tumors specimens were used as positive controls. Negative controls were incubated in negative control reagent.

Evaluation for immunostaining used the HercepTest scoring system. Cases where there was no staining or membrane staining in <10% of tumor cells were scored as 0. Faint/barely perceptible membrane staining in >10% of tumor cells or cells where only part of the membrane stained were scored as 1+. Weak/moderate staining of the entire membrane in >10% of tumor cells were scored as 2+. Strong, complete membrane staining in >10% of tumor cells was scored as 3+. Cases that scored 2+ or 3+ were deemed positive per the HercepTest scoring protocol, whilst those scored 0 or 1+ were deemed negative. Sections were analyzed in a blinded fashion by 2 independent observers and the results of the immunohistochemistry and clinicopathological findings correlated subsequently. Pictures

were acquired with the Leica Assistant Suit (LAS EZ) Software).

EXTRACELLULAR VESICLES ISOLATION

EVs were isolated by size exclusion chromatography (SEC) using Izon qEVoriginal/70nm columns as follows. Conditioned media were collected from cells seeded in 150 mm dishes (seeding density ≈2.5 x 10^6 cells) grown in vitro for 72 hours and concentrated using UFC9100 Amicon® Ultra-15 Centrifugal filters (100 kDa), centrifuged at 6,000 x g for 10 min at 4°C until all media was concentrated. The concentrated sample (500 µl) was added to the Izon column after washing with 10 ml 1X phosphate buffer saline, pH 7.4 (PBS; Boston Bioproducts). Then 15 ml PBS were added, and 30 x 0.5 ml fractions were collected using an Izon automatic fraction collector (AFC). Fractions 7 to 30 were collected for full profile analysis, including western blot. For transfer of EVs, fractions 7-11 were concentrated using Amicon®Ultra-0.5 Centrifugal (30 kDa) to reduce the volume from 2.5 ml to (30-80 µl). For all functional analyses of EVs, each step was performed in a sterile manner (i.e., in fume hoods with UV irradiated filters). All functional experiments were conducted using fresh EVs/protein samples.

EXTRACELLULAR VESICLES TRANSFER EXPERIMENT

Cells were seeded in 96-well culture plates (seeding density 5 x 10^3 cells/well). EVs were added to the cells the day after seeding. 6 hours later, Trastuzumab was added at the indicated concentrations. After 3 days of growth, cell viabilitywas measured by using Cell Titer Glo Luminescent Cell Viability Assay (Promega).

NANOPARTICLE TRACKING ANALYSIS

Number of EVs in PBS was assayed using Nanoparticle Tracking Analysis (NTA) Version 2.2 Build 0375 instrument (NanoSight). Particles were measured for 60 seconds and number of particles (30–800 nm) was determined using NTA Software 2.2. Samples were diluted 1:1000 in PBS prior to analysis.

EXTRACELLULAR VESICLES IN VIVO EXPERIMENTS

1 x 10^6 cells were injected in mice and, after mass formation (average volume 150 mm^3), the

tumors were randomized and treated twice, 24 hours apart, with the indicated extracellular vesicles (≈ 1×10^{11} total number of EVs/tumors). After another 48 hours the tumors were treated with either vehicle (saline per os) or Trastuzumab (30 mg/ kg, weekly ip). Tumor size was evaluated once weekly by caliper measurements and approximate volume of the mass was calculated using the formula $4/3\pi(D/2)(d/2)2$, where D is the major tumor axis and d is the minor tumor axis.

THE SCIENTIFIC PROBLEM AND THE AIM OF THE WORK

Over the past decade, the development of targeted therapies and the optimization of existing chemotherapeutics have expanded treatment options for advanced GC and ensured better survival expectations for patients. At the same time, global efforts are being made to study in detail the genomic and epigenomic heterogeneity of this disease, identify new specific and sensitive predictive and prognostic biomarkers and carry out innovative molecular classifications based on gene expression profiles. Nonetheless, several randomized trials aimed at exploring new and innovative drugs have failed to demonstrate clinically significant survival advantages. Therefore, it is essential to further investigate the reasons behind these decades-long negative results.

Aim of my PhD work was to understand why most anti-HER2 antibodies and tyrosine kinase inhibitors have failed in clinical trials in gastric cancer for HER2+ patients with advanced disease and how even Trastuzumab, the only approved HER2 target therapy approved, is affective only in a low percentage of patients theoretically suitable for this treatment.

Hence the focus of the project was on different aspects of the role of HER2: i) definition of a new and more accurate selection of patients to increase drug efficacy; ii) evaluation of the importance of the tumor mutational burden, the microenvironment, and the extracellular vesicles in sustaining drug resistance. These results have the purpose of improving the molecular characterization of GC subgroups, in order to provide researchers and medical oncologists with new tools for patients' selection and stratification in future clinical development programs and subsequent trials.

RESULTS

1. THE "GEA PLATFORM" PROJECT

The tumors studied in this work were selected based on their molecular characteristics from a large collection of gastro-esophageal PDXs, generated in the frame of the GEA (Gastro-Esophageal cancer Annotated Platform) project. This project aims at collecting and characterizing at molecular level a wide number of gastro-esophageal cancers in order to i) identify new molecular targets; ii) optimize molecular therapies; iii) identify biomarkers of response/resistance to molecular therapies. This platform includes tumors collected from 15 different Italian hospitals between 1/11/2011 and 31/8/2019. This collection was established by processing *ad hoc* surgically removed tumor tissue specimens for pathologic and molecular characterization and by transplanting small tumor fragments (roughly 4mm x 4mm) into immunocompromised mice (NOD SCID), in order to obtain a molecular characterization of the original tumor and, at the same time, to generate PDXs to perform xenotrials.

2. PATIENTS' CHARACTERISTICS

Samples which gave origin to this platform were collected from 570 gastro-esophageal cancer patients. Men were twofold more represented than women (2:1) and the median age was 68 years. 16% of total tumors were classified as gastro-esophageal junction cancers, and 5%, 23%, 48% as cancer of the fundus, body, and antrum/pylorus, respectively. According to Lauren's classification, intestinal subtype was the most represented (66%), whereas diffuse and mixed types were present in 29% and 5% of cases, respectively. 39% of patients were diagnosed at stages I/II and 61% at stages III/IV (115).

3. ESTABLISHMENT OF PDX MODELS

Tumor samples included in this platform were divided in a variable number of small fragments, according to their dimensions, and each fragment was implanted in a different immunocompromised mouse. Success rate of engraftments was around 30%, in line with the literature. The mean latency period of tumor growth (from implant to the appearance of a palpable tumor) was 73 days (ranging from 31 to 200). This latency period became progressively shorter in the following serial passages (a mean of 3 weeks was necessary for the growth of the tumors used in xenotrials). Histological analysis of tumors grown in mouse models revealed features comparable to their corresponding original tumors (grown in human patients) (115).

4. FACTORS INFLUENCING PDX GENERATION

No statistically significant correlation between engraftment and patient characteristics (such as age, gender, or neoadjuvant chemotherapy) was observed by analyzing both patient and PDX features.

Tumors classified as intestinal showed a better grafting (66% of primary tumors were of this subtype versus 84% of PDXs) than those of diffuse type (29% versus 12%) or the ones with mixed type (5% versus 4%), demonstrating that histological characteristics were associated with the success of engraftment. The same was true for the MSI status: 18% of primary tumors were MSI+ versus 24% of the PDXs. Conversely, tumor location, TNM stage and EBV status did not correlate with engraftment.

Receptor tyrosine kinases (namely HER2, EGFR, MET and FGFR2) and KRAS amplifications significantly correlated with tumor engraftment: in fact, 20% of PDXs presented RTK high level (>8 copies) of amplification, which was observed only in 13% of primary tumors. In particular, 5% of primary cancers were amplified for HER2, versus 9% of PDXs (Figure 15A) (115).

5. PDX CHARACTERIZATION

PDX histological analyses were routinely performed and demonstrated a good correlation with the corresponding original tumor (Figure 15B). Also, MSI and EBV status remained stable between primary and PDXs samples (*data not shown*). RT-PCR analyses were performed for each specimen, in order to test and confirm gene amplifications in PDX tumors (115).

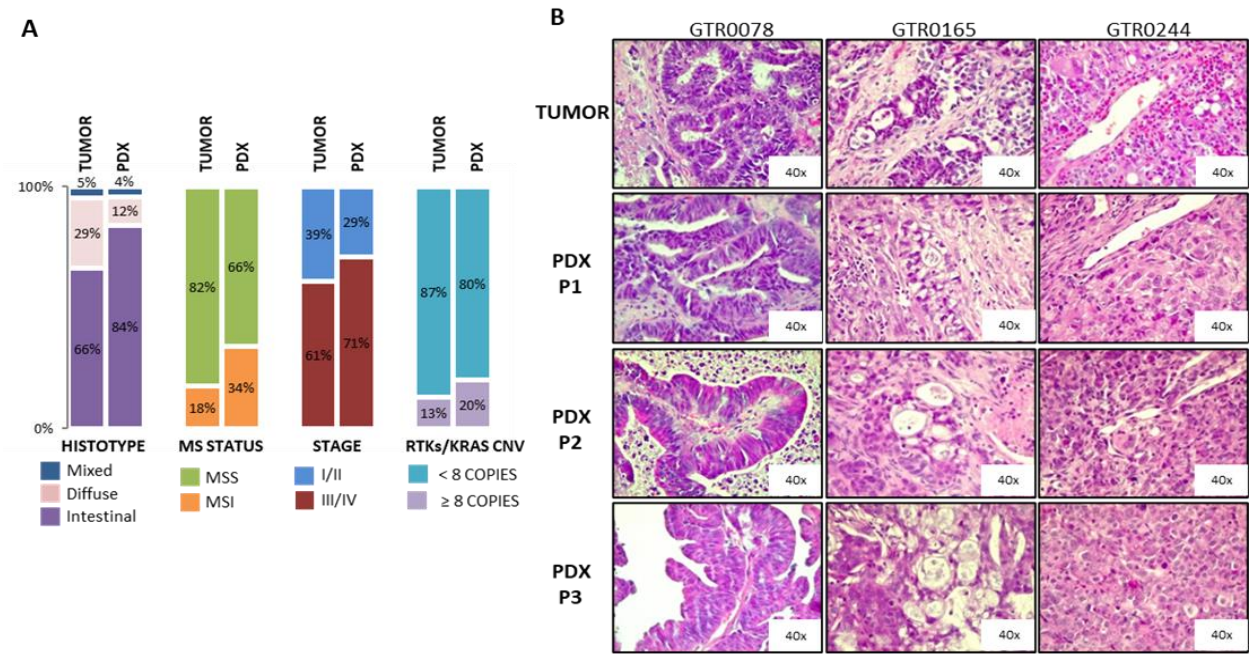


Figure 15: The PDX platform captures all the GC subtypes, and it is enriched in intestinal histology, MSI status, high stage and RTKs/KRAS amplification compared to donor tumours. **A**. The graph illustrates the percentage of donor tumours and of derived PDXs for the following features: histology (intestinal, diffuse, or mixed); MS status (MSI or MSS); stage (I/II or III/IV); RTK/KRAS copy number variation (CNV < or ≥8 copies). **B**. Representative micrographs of three gastric adenocarcinomas featuring distinct growth-patterns. GTR0079 is a moderately differentiated gastric adenocarcinoma of intestinal type, showing a glandular architecture; the intestinal type GTR0165 adenocarcinoma also displays foci of mucin production; GTR0244 shows a diffuse growth-pattern. As illustrated, xenografted tumors retained the histopathologic characteristics of the original samples through passages.

6. PREVALENCE OF HER2 AMPLIFICATION

Molecular characterization of 570 GCs allowed the identification of 27 tumors which carried an altered number of HER2 gene copies in this platform (Table 2). From the 570 samples, we were able to generate PDXs in 151 cases. Among them, 8 PDXs were bearing ≥ 8 HER2 gene copies. We called this selected subgroup HER2 "hyper"-amplified. They represented 5% of the recruited cases and 9% of engrafted tumors. Primary tumors were preferentially located in the pyloric region and were of the intestinal subtype, according to Lauren's classification. Curiously, diffuse subtype was more represented among those tumors with a higher number of HER2 copies. Since at the moment there are no indications to define the most appropriate criteria to select patients who could benefit from HER2 targeting, for this study we decided to consider only tumors carrying at least 8 HER2 copies, as this number has been shown to represent the threshold of sensibility for therapies targeting HER2 (178). FISH analysis confirmed HER2 gene amplification and IHC analysis revealed that all the models were HER2 3+. In the selected tumors, we observed more than 90% HER2 positive tumor cells (Figure 16A). Importantly, the histopathologic features of the PDXs recapitulated those of the tumors of origin (Figure 16B).

TUMOR with *HER2* CNG (>8 COPIES)	HER2 copy number in TUMOR (2 TUMOR AREAS)	*HER2* copy number in PDX	Tumor site	Histology Laureen Classification (centralized)	Histology WHO Classification	Grade	Stage	IHC Hercep Test	*EGFR/MET /KRAS/FGF R2* CNG in PDX (>8 COPIES)	MSI Status	EBV	RELAPSE	TRASTUZUMAB-CONTAINING TREATMENT	RESPONSE TO TASTUZUMAB-CONTAINING TREATMENT	INDIVIDUAL PFS (months)
GTR0031	6-8	10	antrum	intestinal	tubular	G2	IIB	3+	KRAS	WT	NEG	No	N/A	N/A	N/A
GTR0034	9-11	NO PDX	antrum/ pylorus	intestinal	tubular	G2	IIB	3+	N/A	MSI	NEG	No	N/A	N/A	N/A
GTR0048	4-8	6	antrum/ pylorus	intestinal	tubular	G2	IIIB	3+	NEG	WT	NEG	YES	NO	N/A	N/A
GTR0108	130-170	200	antrum	intestinal	tubular,	G2	IIIA	3+	NEG	WT	NEG	No	N/A	N/A	N/A
GTR0109	3-9	8	fundus	intestinal	tubular	G3	IV	3+	NEG	WT	NEG	YES	ND	N/A	N/A
GTR0117	360-400	NO PDX	corpus	intestinal	tubular	G3	IIIC	3+	N/A	WT	NEG	YES	NO	N/A	N/A
GTR0121	7-8	NO PDX	ND	intestinal	tubular	G3	IB	2+	N/A	WT	NEG	NO	N/A	N/A	N/A
GTR0152	11-16	NO PDX	antrum/ pylorus	intestinal	tubular	G2	IIIB	3+	N/A	WT	NEG	NO	N/A	N/A	N/A
GTR0167	2-9	2	antrum	intestinal	tubular	G2	IIB	3+	NEG	WT	NEG	NO	N/A	N/A	N/A
GTR0233	NA	50	cardia	ND	ND	G2	IIA	3+	NEG	WT	NEG/ LOW	NO	N/A	N/A	N/A
GTR0238	44-60	NO PDX	corpus	intestinal	tubular, papillary	G2	IIIC	3+	N/A	WT	NEG	NO	N/A	N/A	N/A
GTR0257	NA	15	antrum	intestinal	tubular	G3	IIA	2+;	NEG	WT	NEG	YES	YES	SD	3
GTR0277	220-250	300	fundus	intestinal	papillary	G3	IIB	3+	NEG	WT	NEG	YES	NO	N/A	N/A
GTR0290	8*	NO PDX	cardia	intestinal	tubular	G2	IIA	2+	N/A	WT	NEG	NO	N/A	N/A	N/A
GTR0301	3-40	NO PDX	cardia	ND	poorly cohesive	G3	IIIB	NEG	N/A	WT	NEG	NO	N/A	N/A	N/A
GTR0357	120*	NO PDX	cardia	intestinal	papillary	ND	IIB	3+	N/A	WT	NEG	YES	NO	N/A	N/A
GTR0372	18*	NO PDX	antrum/ pylorus	intestinal	mucinous	G3	IIB	ND	N/A	WT	NEG	YES	NO	N/A	N/A
GTR0374	10-56	NO PDX	antrum/ pylorus	intestinal	papillary	G2	IIIB	3+	N/A	WT	NEG	YES	YES	PR	15
GTR0402	28-40	68	antrum/ pylorus	mixed	papillary	G2	IIIB	3+	NEG	WT	NEG	YES	YES	PR	51
GTR0402_ METS	40*	80	Lung mets	-	-	G2	-	-	-	-	-	-	-	-	-
GTR0426	8*	NO PDX	cardia	mixed	tubular, papillary	G2-G3	III	3+	N/A	WT	NEG	YES	YES	PR	16
GTR0435	18-100	2	corpus	diffuse	tubular, papillary	G3	IV	3+	NEG	WT	NEG	NO	NO	N/A	N/A
GTR0455	ND	80	cardia	intestinal	tubular	G3	IV	3+	NEG	WT	ND	YES	YES	PD	N/A
GTR0471	ND	8-10	ND	ND	ND		ND	ND	NEG	WT	ND	YES	YES	SD	5
GTR0496	13-30	NO PDX	ND	ND	ND	G3	ND	2+; 3+	N/A	MSI	NEG	YES	YES	SD	24
GTR0528	50-150	NO PDX	curpus	intestinal	tubular	G3	IV	3+	N/A	WT	ND	YES	YES	PD	N/A
GTR0538	2-200	NO PDX	cardia	ND	tubular	ND	IIB	ND	N/A	WT	NEG	YES	NO	N/A	N/A

Table 2. Molecular, histological and pathological features of HER2 "hyper"-amplified tumors (≥8 HER2 gene copies in at least one tumor area), and patients' response to trastuzumab-containing treatments (PD=progressive disease; SD= stable disease; PR=partial response, according to RECIST 1.1). ND= Not Determined; N/A= Not Applicable; *only one tumor area analyzed.

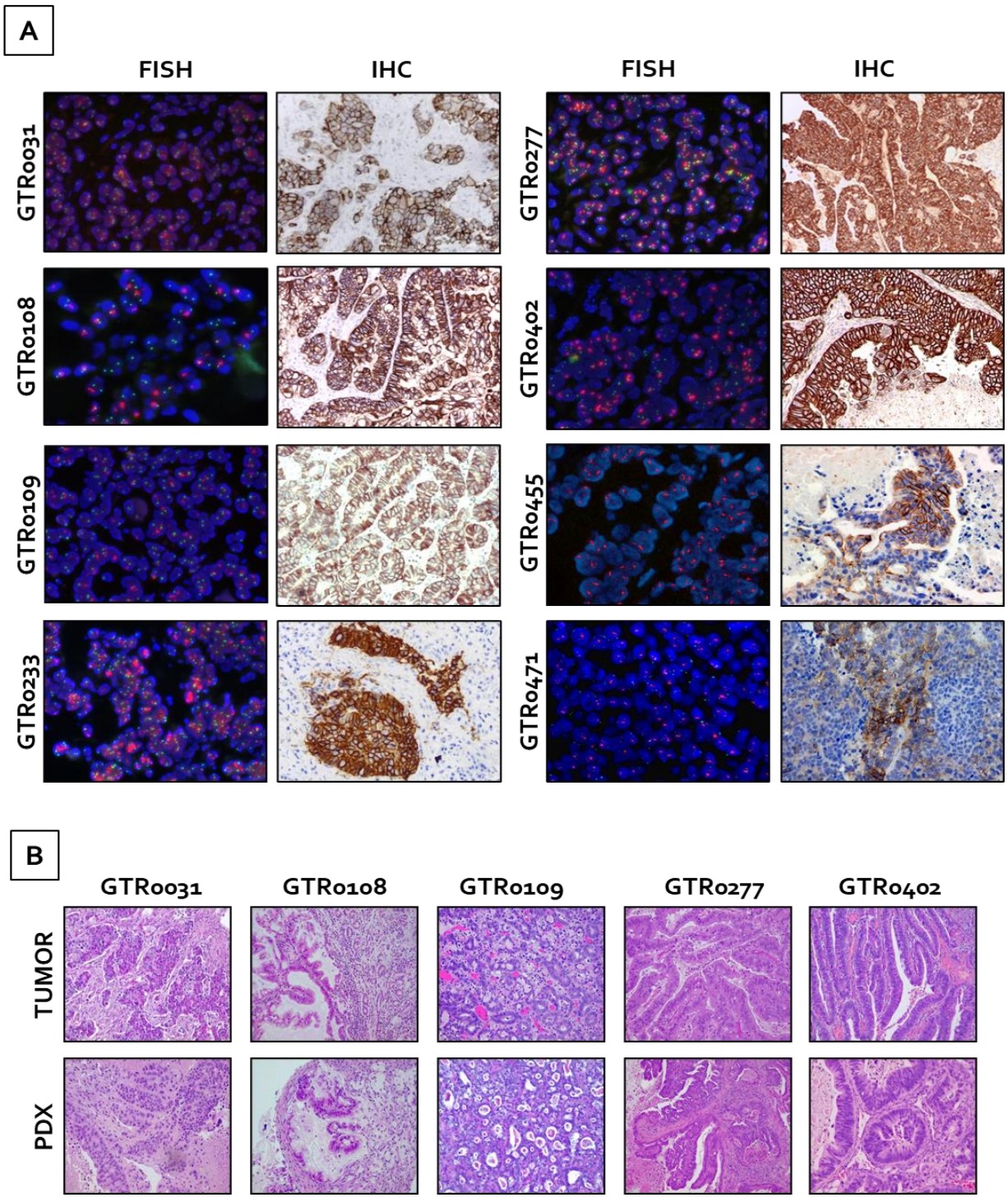
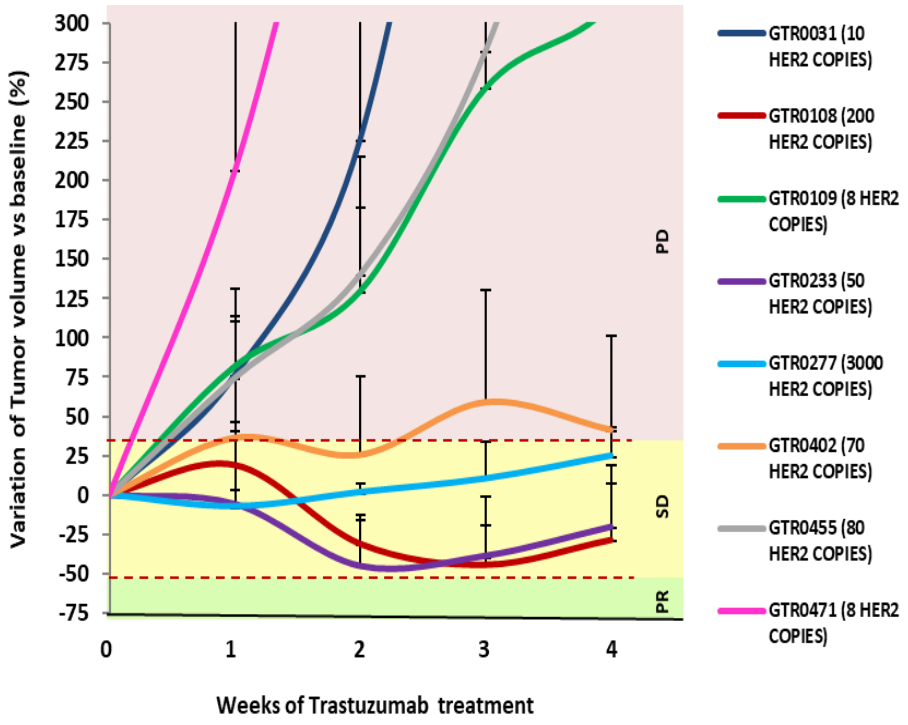


Figure 16: **A.**HER2 amplification / expression in HER2 "hyper"-amplified GC PDXs. Microphotographs of FISH (left panels, 40x magnification) and IHC (right panels, 20X magnification) performed on slices derived from the indicated PDXs. For FISH analysis the HER2 probe is labelled in red, the CH17 centromere probe is labelled in green. **B.** Comparison between available primary tumors and the derived PDXs. Microphotographs of H&E staining of the primary tumors (upper line) and the corresponding PDXs (lower line).

7. RESPONSE TO TRASTUZUMAB

In our trial, we used Trastuzumab (a recombinant humanized IGG1 monoclonal antibody against the HER2 ectodomain, inhibiting ligand independent HER2 activation), because it is the only therapy targeting molecular alterations so far approved in gastric cancer for HER2+ patients with advanced disease. However, clinical experience shows that only a small fraction of HER2 amplified patients benefit from Trastuzumab treatment (< 20%). Therefore, we evaluated the effect of this Mab in our models that, at least in principle, had the highest probability of being Trastuzumab sensitive due to the high level of HER2 amplification (179). These 8 PDX models were passaged in vivo until five tumor-bearing animals/treatment group were produced, to evaluate the effect of HER2 inhibition. When xenografts reached an average volume of ~ 250 mm^3, mice were treated with Trastuzumab, and tumor response was evaluated according to RECIST-like Criteria (177).

It is important to underline that these experiment evaluated the effect of pure inhibition of the HER2 pathway, without the confounding effect of chemotherapy and of the activity of the immune system, since the NGS mice almost completely lack the immune system.



As shown in figure 17, only 4 out of the 8 models displayed a clinical response to Trastuzumab, including two stable diseases (SD, GTR0277 and GTR0402) and two partial responses (PR, GTR0108 and GTR0233).

Figure 17: Response to Trastuzumab treatment in PDXs bearing high HER2 copy number gene (CNG). The Spaghetti plot illustrates the effect of trastuzumab treatment (30 mg/kg) on PDXs with a HER2 CNG ≥ 8 copies. Individual lines represent, for each PDX model, the mean percentage variation in tumor burden, from treatment start (day 0) to 4 weekly consecutive serial assessments (N = 5 mice for each model). Tumor response has been evaluated using RECIST 1.1-like (177): progressive disease (PD): ≥ 35% increase from baseline (pink background); partial response (PR): ≥ 50% reduction from baseline (green background); stable disease (SD): intermediate variations from baseline (yellow background).

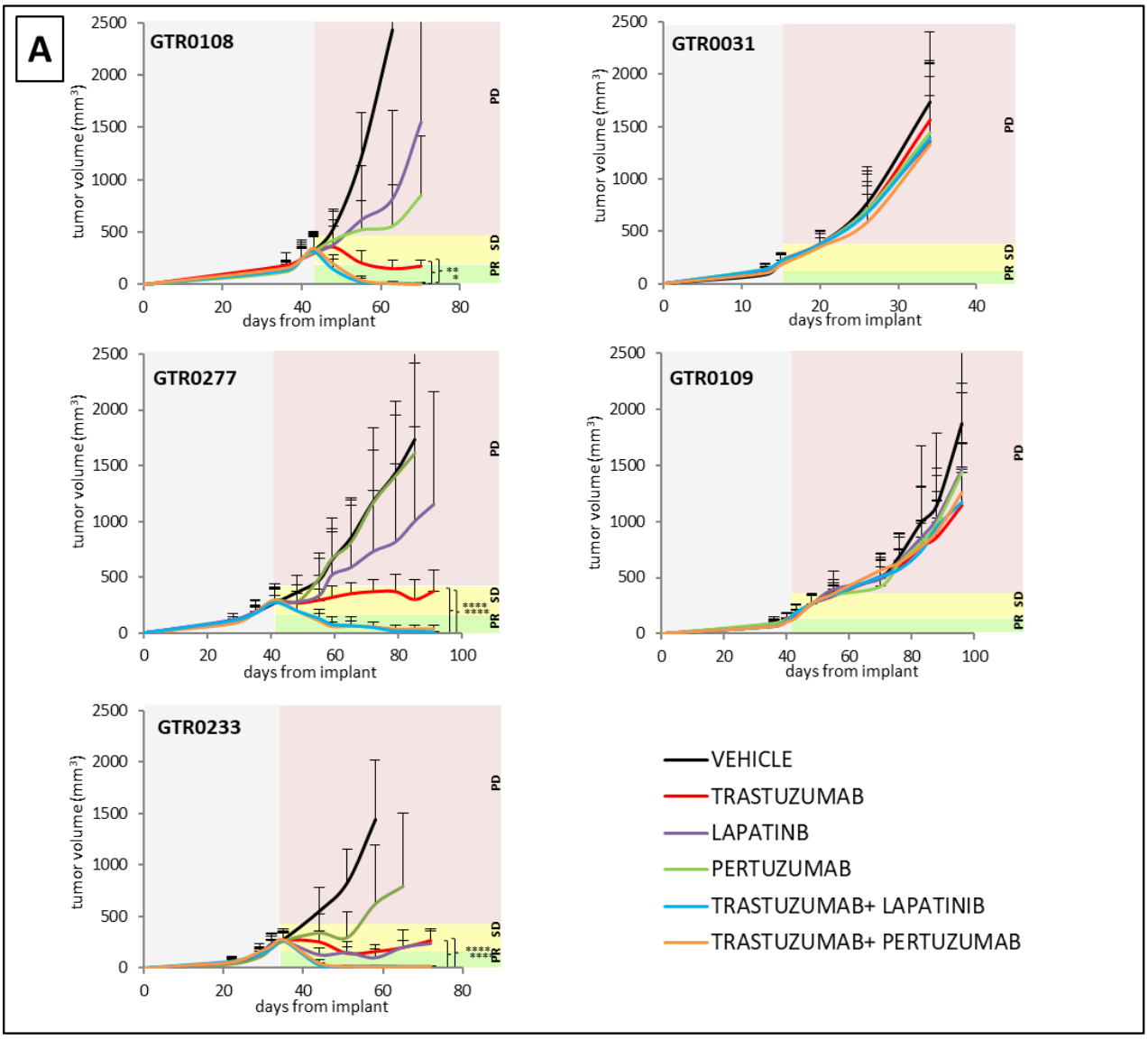
8. TRASTUZUMAB COMBINATORIAL THERAPIES ARE MORE EFFECTIVE THAN TRASTUZUMAB MONOTHERAPY

Since many clinical trials, in different tumor contexts such as breast and colorectal cancer, have demonstrated that dual HER2 blockade increases the pathologic complete response rate (180) (181) (182), we evaluated whether dual-HER2 blockade might improve the efficacy in terms of response compared to Trastuzumab monotherapy in HER2 "hyper"-amplified tumors (HER2+ PDXs scoring 3+ at the IHC HercepTest and bearing ≥ 8 HER2 copies). Therefore, we tested different HER2-targeted drugs or their combinations. The different treatment groups were: (1) Trastuzumab ("gold standard"); (2) Pertuzumab (a humanized monoclonal antibody directed against a different epitope in the extracellular subdomain 2 of HER2, inhibiting ligand-dependent heterodimerization of HER2 with other HER family members); (3) Lapatinib (dual HER2/EGFR TKI), (4) Trastuzumab plus Lapatinib; (5) Trastuzumab plus Pertuzumab; (6) vehicle. To evaluate the pure response to HER2 inhibition, mice did not receive any chemotherapy. As shown in figure 18A, Trastuzumab monotherapy led to 2 PR and 1 SD (GTR0108, GTR0233 and GTR0277, respectively); Pertuzumab monotherapy had no therapeutic efficacy, while Lapatinib achieved PR only in the GTR0233 PDX. In 3 out of 5 cases (GTR0108, GTR0233, GTR0277, displaying 200, 50 and 300 HER2 gene copies, respectively), Trastuzumab plus Pertuzumab or Lapatinib was significantly more effective than Trastuzumab monotherapy, resulting in complete responses (CR) in 3 out of 3 cases. Interestingly, in the GTR0277 model, we also identified a HER3 activating mutation (p.G284R) that could be responsible for the relatively low sensitivity to Trastuzumab monotherapy (183). Indeed, the dual-HER2 block, interfering with

heterodimers formation and activation, led to a complete response (figure 18A). From this PDX, we derived in vitro primary cells which maintained both HER2 amplification and the HER3 mutation. *In vitro* experiments showed that combinatorial treatment with Trastuzumab plus an anti-HER3 MoAb (MM-121/Seribantumab) resulted in a strong growth inhibition *(data not shown).*

In 2 PDX models (GTR0108 and GTR0233), we performed long-term treatment to evaluate the possible onset of secondary resistance to the mono and combo treatments. As shown in figure 18B, while resistance to Trastuzumab monotherapy invariably emerged, we never observed tumor reappearance in animals treated with dual-HER2 blockade combinations. Even more strikingly, in the combo-treated mice, we did not observe tumor regrowth upon drug removal, meaning that the treatment could be regarded as curative. Notably the prolonged dual treatment did not result in any overt toxicity (*not shown*).

These results suggest that dual-HER2 blockade improves the efficacy in terms of intensity and duration of response, compared to Trastuzumab monotherapy in HER2+ PDXs scoring 3+ at the IHC HercepTest and bearing ≥ 8 HER2 copies.


A
GTR0108
GTR0031
GTR0277
GTR0109
GTR0233
tumor volume (mm³)
days from implant
PD
PR
SD
VEHICLE
TRASTUZUMAB
LAPATINB
PERTUZUMAB
TRASTUZUMAB+ LAPATINIB
TRASTUZUMAB+ PERTUZUMAB

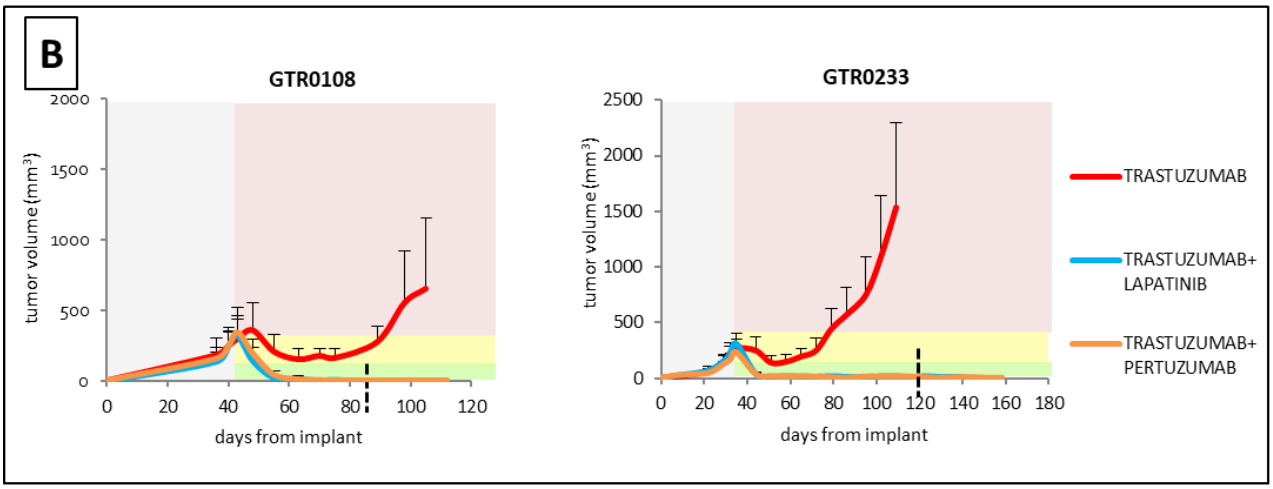

B
GTR0108
GTR0233
tumor volume (mm³)
days from implant
TRASTUZUMAB
TRASTUZUMAB+ LAPATINIB
TRASTUZUMAB+ PERTUZUMAB

Figure 18: Dual HER2 blockade is more effective and durable than Trastuzumab monotherapy in HER2-hyper-amplified PDXs. **A.** Tumor growth curves of mice cohorts derived from GTR0108, GTR0233, GTR0277, GTR0031 and GTR0109 patients, treated with the HER2 inhibitors Trastuzumab, Pertuzumab or Lapatinib, alone or in combination, as indicated. Grey background: growth of the tumors before treatment start. The response in mice has been evaluated using RECIST 1.1-like criteria, progressive disease (PD): ≥ 35% increase from baseline (pink back- ground); partial response (PR): ≥ 50% reduction from baseline (green background); stable disease (SD): intermediate variations from base- line (yellow background). Complete Response (CR): 100% reduction from baseline. **B.** Tumor growth curves of mice cohorts derived from GTR0108 and GTR0233 PDXs undergoing prolonged (> 6 weeks) treatment with Trastuzumab, or with the combos Trastuzumab + Lapatinib or Trastuzumab + Pertuzumab. Grey background: tumor growth before treatment start. The response in mice has been evaluated using RECIST 1.1-like criteria, as in A. The dashed line indicates stop of combo treatments. Mice receiving trastuzumab monotherapy continued the treatment until the end of the experiment or until mice were sacrificed for the tumor size. N = 5 mice (GTR0108, GTR0233, GTR0277); N = 6 mice (GTR0031; GTR0109); data are represented as mean + SD; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Two-way ANOVA followed by Bonferroni multiple comparisons test has been used

9. DUAL HER2 TARGETING THERAPY STRONGLY INHIBITS HER2-DEPENDENT SIGNAL TRASDUCTION

To investigate which pathways were inactivated by the different drugs/drug combinations, we performed biochemical studies on the available PDX-derived primary cells. GTR0233 and GTR0277 cells (in which HER2 amplification was confirmed by RT qPCR) were treated with Trastuzumab and Lapatinib, alone or in combination. Viability assays showed that also *in vitro*, the combo treatment was significantly more effective than each drug used in monotherapy (figure 18A, B, left part). Western blot analysis showed that while monotherapy with either Trastuzumab or Lapatinib poorly affected activation of downstream transducers, such as AKT, MAPK and S6 (evaluated as read out of the PI3K, RAS/MAPK and mTOR pathways, respectively), the drug combination resulted in a strong inhibition of signal transduction (figure 19A, B, right part). Very similar results were obtained with organoids derived from the GTR0108 PDX *(not shown).* These *in vitro* findings strongly support the results obtained in the *in vivo* experiments where Trastuzumab induced only SD or PR, while dual-HER2 blockade resulted in durable CRs.

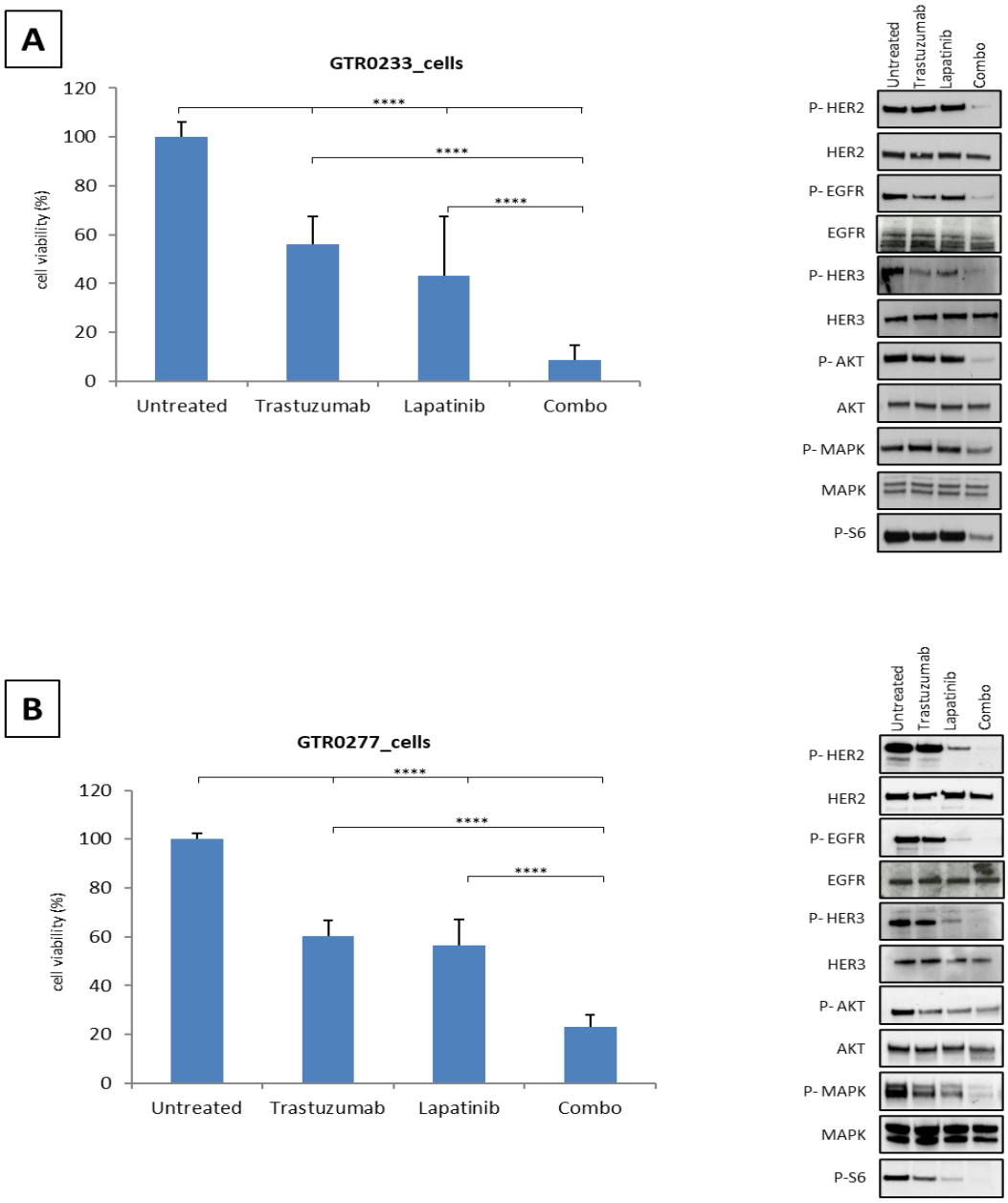


Figure 19: Dual HER2 blockade is more effective than Trastuzumab alone in GTR0233 and GTR0277 PDX-derived cells in vitro. Cell viability assay performed on GTR0233 (**A,** left panel) and GTR0277 (**B,** left panel) tumor-derived cells, upon treatment for 6 days with the indicated drugs at IC50 for each cell type (GTR0233: Trastuzumab 0.15 µg/ml; Lapatinib 1 nM; GTR0277: Trastuzumab 10 µg/ml; Lapatinib 10 nM). Western blot analyses showing the activation state of HER2, EGFR and their downstream targets (AKT, MAPK and S6) in GTR0233 (**A,** right panel) and GTR0277 (**B,** right panel) tumor-derived cells treated for 24 hours with the indicated drugs/ drug combinations (same doses used in the cell viability assays). Data are represented as mean of biological triplicates + SD; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. One-way ANOVA followed by Dunnett multiple comparisons test has been used.

11. LACK OF RESPONSE TO ANTI-HER2 TREATMENTS IN CELL LINES WITH <8-10 HER2 COPIES

Intriguingly, we noticed that the two PDX models presenting 8–10 HER2 copies (namely GTR0109 and GTR0031) did not show response to Trastuzumab and did not get any benefit from the combo treatment (figure 17A). While genomic analysis of the GTR0031 model revealed the presence of KRAS co-amplification (8 copies), known to be responsible for resistance to targeting of other RTKs in different tumor contexts (74) (184), no putative genomic alteration likely sustaining Trastuzumab resistance was identified in GTR0109. We thus wondered if the level of HER2 amplification observed in these two PDXs could not be sufficient to render HER2 a real oncogenic driver in these cells. We thus tested *in vitro* if HER2-positive GC cell lines (primary and established) with less than 10 gene copies were resistant to HER2 targeted treatments. As shown in Figure 20A, cells displaying less than 10 HER2 copies did not respond neither to Trastuzumab nor to the combo, while all the "hyper" amplified cells (both primary and established) showed a response to Trastuzumab, further improved by the combo.

We also performed an in vivo trial on an available model, GTR0471, displaying the same range of HER2 copies (8-10). As for the other two cases presenting a similar level of HER2 amplification, we did not observe response to neither Trastuzumab nor combos (Figure 20B).

Even if a larger cohort of cases is needed, the presented data, *in vitro* and *in vivo*, further reinforce the idea that a level of HER2 amplification higher than 8–10 copies is required for HER2 to be considered as a driver of oncogene addiction.

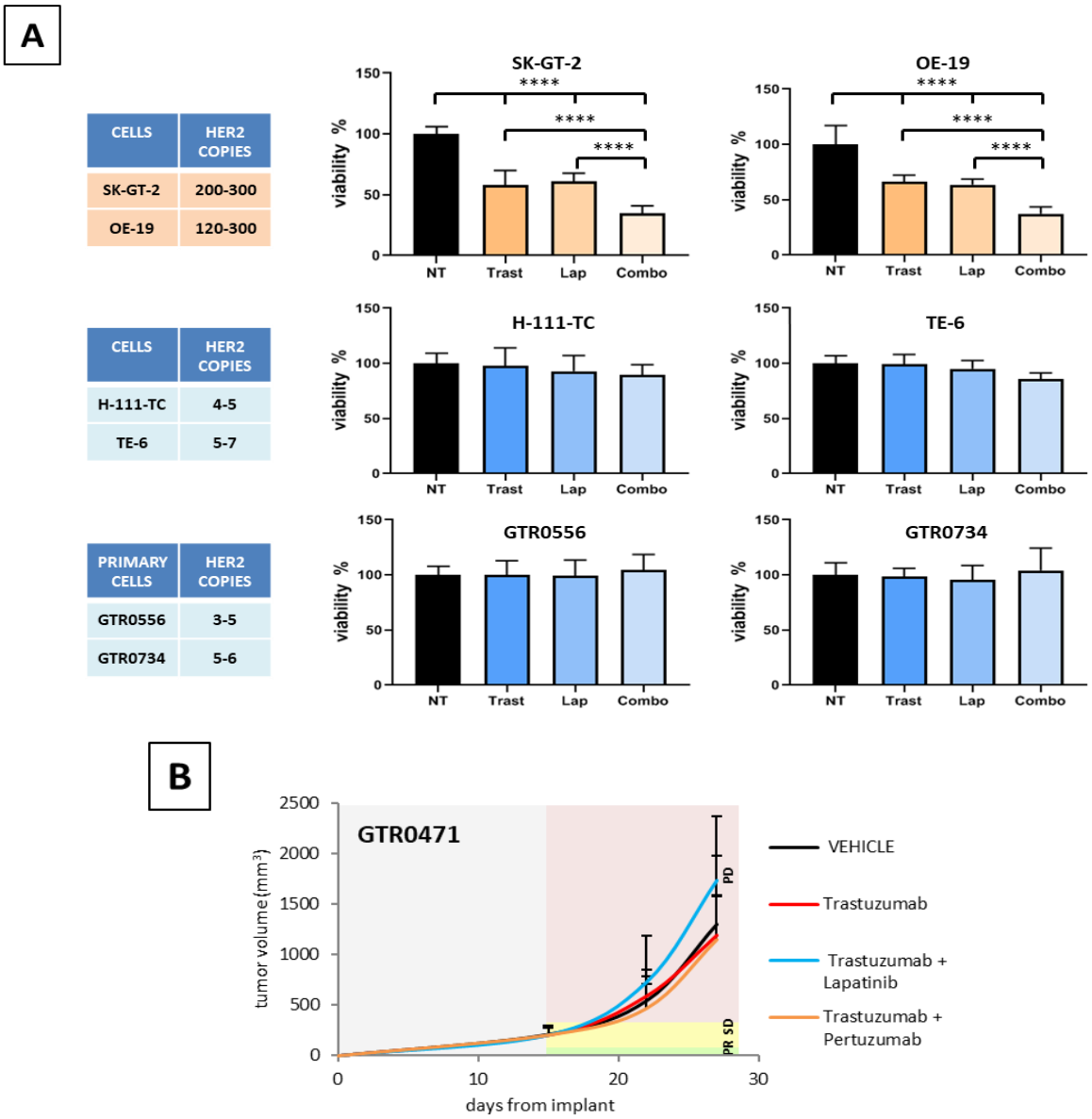


Figure 20: Lack of response to anti-HER2 treatments in cell lines and PDXs with <8 HER2 copies. **A.** Cell viability assay performed on commercial cell lines bearing ≥ 8 HER2 copies (upper panels) or <8 HER2 copies (middle panels), or on HER2 primary cell lines with <8 HER2 copies (lower panels). Cell were treated for 3 days (commercial cell lines, plated in low attachment) or 6 days (primary cells), with the indicated drugs (Trastuzumab 10 µg/ml; lapatinib 25 nM). Data are represented as mean of biological triplicates + SD; ****p <0,0001. One-way ANOVA followed by Dunnett multiple comparisons test has been used. **B**. Tumor growth curves of mice cohorts derived from GTR0471 tumor, treated with Trastuzumab, alone or in combination with Lapatinib or Pertuzumab, as indicated. Grey background: growth of the tumors before treatment start. The response in mice has been evaluated using RECIST 1.1-like criteria, as in Figure 17.

11. ACTIVITY OF TRASTUZUMAB-CONJUGATES IN TRASTUZUMAB-RESISTANT PDXs

Even if the data obtained, *in vitro* and *in vivo*, highlighted that an amplification level of HER2 higher than 8-10 copies is necessary for HER2 to be classified as an oncogene driver, we evaluated the possible use of the HER2 receptor as a docking molecule for Antibody Drug Conjugates (ADC). Since T-DM1, a humanized MoAb Trastuzumab covalently linked to the cytotoxic agent DM1, was shown to be effective in breast cancer (185), we investigated whether it could overcome Trastuzumab resistance in the two non-responsive PDXs GTR0109 and GTR0031. As shown in figure 21A, T-DM1 effectively inhibited GTR0109 (SD), but it was inactive in GTR0031. The new ADC Trastuzumab deruxtecan (DS-8201a, consisting of Trastuzumab covalently linked to the topoisomerase inhibitor deruxtecan), showing activity in patients with heavily pre-treated HER2-positive GC (72), has recently been approved by FDA and the Japanese Ministry of Health, Labor, and Welfare for the treatment of patients with HER2-positive unresectable advanced or recurrent gastric cancer (186) (187). We administered this agent to GTR0031 PDXs. As displayed in figure 21B, DS-8201a induced a CR in this PDX, refractory to Trastuzumab, dual-HER2 blockade and T-DM1.

These findings provide the biological rationale for the use of HER2-directed ADCs to efficiently treat also those tumors displaying either primary or acquired Trastuzumab resistance.

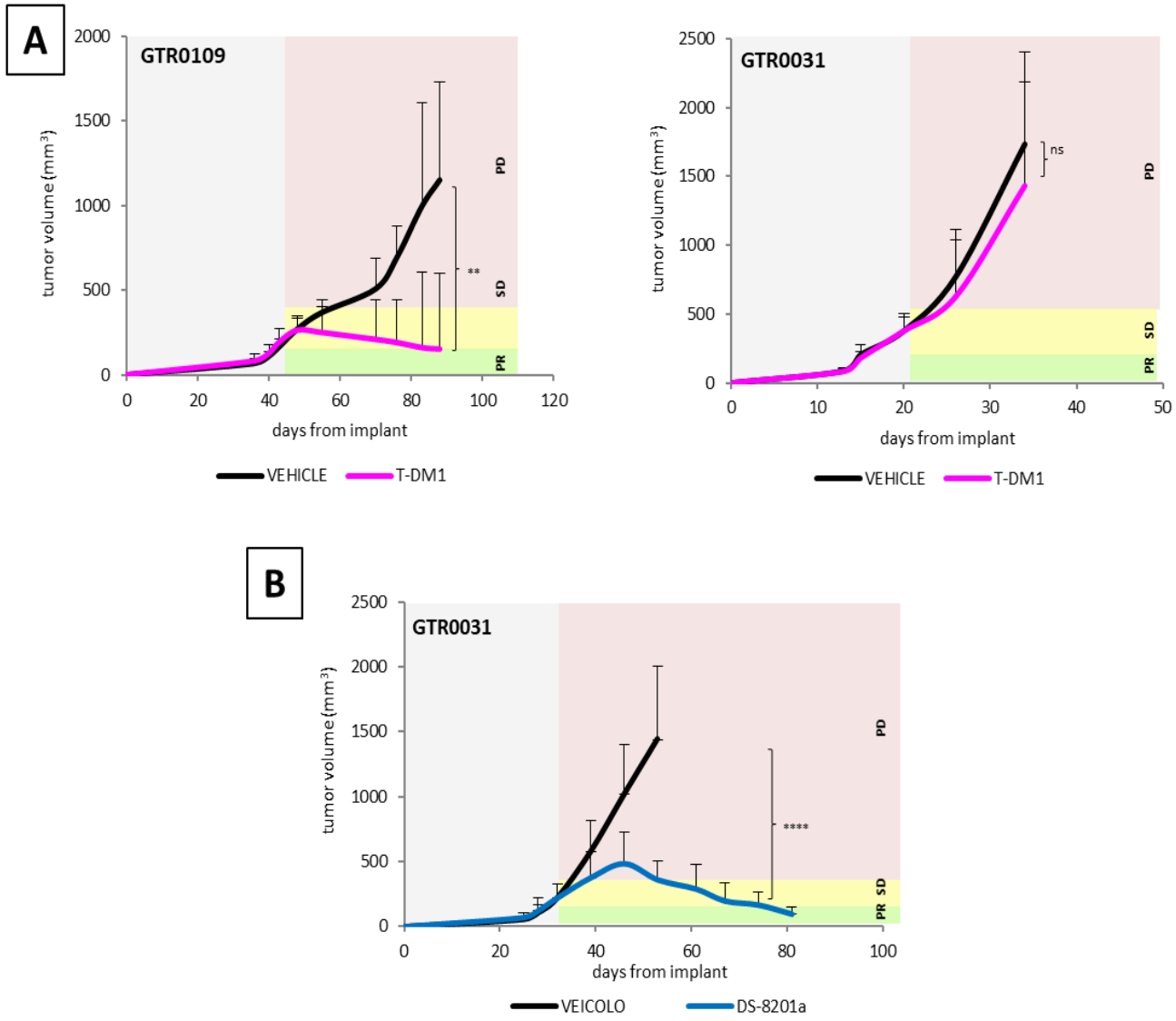
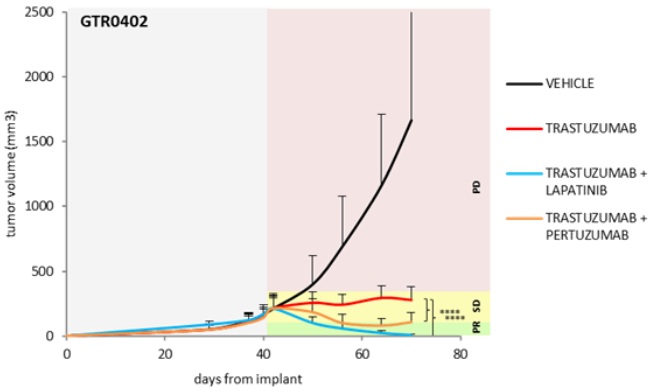


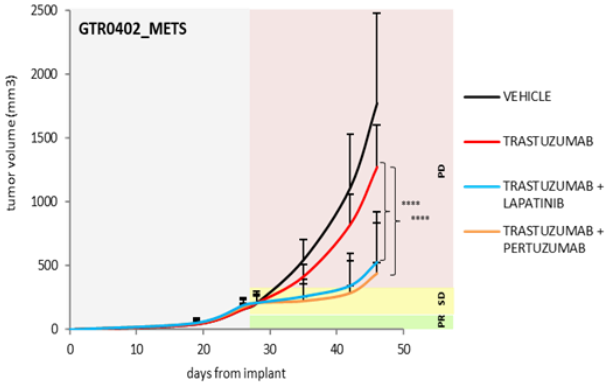
Figure 21: Activity of trastuzumab-conjugates in resistant PDXs. **A**. Tumor growth curves of mice cohorts derived from GTR0109 (left panel) and GTR0031 (right panel) treated with the HER2 antibody conjugate T-DM1. Grey background: growth of the tumors before treatment start. The response in mice has been evaluated using RECIST 1.1-like criteria, as in figure 18. **B**. Tumor growth curves of mice cohorts derived GTR0031 treated with the HER2 antibody conjugate DS-8201a. The response in mice has been evaluated using RECIST 1.1-like criteria, as in figure 18. Data are represented as mean + SD; ns= not significant; **p <0,01; ****p <0,0001. Two tail student t-test has been used.

12. PATIENT-DERIVED XENOGRAFT MODELS RECAPITULATE PATIENTS' RESPONSE TO TRASTUZUMAB

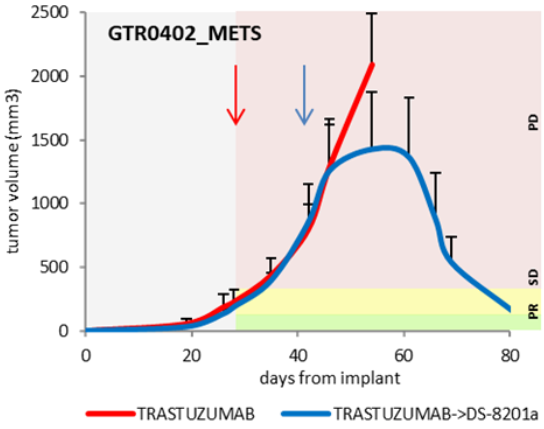
Only two PDXs (namely GTR0402 and GTR0455) of our GC platform derived from patients who received a Trastuzumab-containing therapy. The patient originating the GTR0402 PDX, after tumor removal, received first a chemo + trastuzumab regimen, leading to PR, and later Trastuzumab monotherapy as maintenance, resulting in a prolonged SD. In the GTR0402 PDX model derived from the primary gastric adenocarcinoma (68 HER2 copies), we observed SD in response to trastuzumab (figure 22A, left part), similar to what was determined by Trastuzumab monotherapy in the patient. In this PDX model, we also evaluated whether (as observed in GTR0277, GTR0233 and GTR0108 models) the response could be improved by the addition of either Lapatinib or Pertuzumab. Xenografts were thus randomized into 4 cohorts and treated with (1) vehicle; (2) Trastuzumab; (3) Trastuzumab + Lapatinib; (4) Trastuzumab + Pertuzumab. As reported in figure 22A, the combos overperformed compared to trastuzumab monotherapy, leading to either PR (Trastuzumab + Pertuzumab) or CR (Trastuzumab + Lapatinib). From one lung metastasis resected at patient progression, we could derive another PDX model (GTR0402_METS; 80 HER2 copies, that was expanded and randomized in the same cohorts as the PDX derived from the primary tumor (figure 22A, right part). Interestingly, PDXs derived from the metastatic tumor were not responsive to Trastuzumab, mimicking again the patient's response. Even in this setting, the two combos (Trastuzumab + Lapatinib and Trastuzumab + Pertuzumab) performed better than Trastuzumab alone, inducing a temporary stabilization of disease. A much stronger response was induced by DS-8201a which led to tumor regression proving the activity of this drug conjugate also in the context of acquired resistance (figure 22B).

A

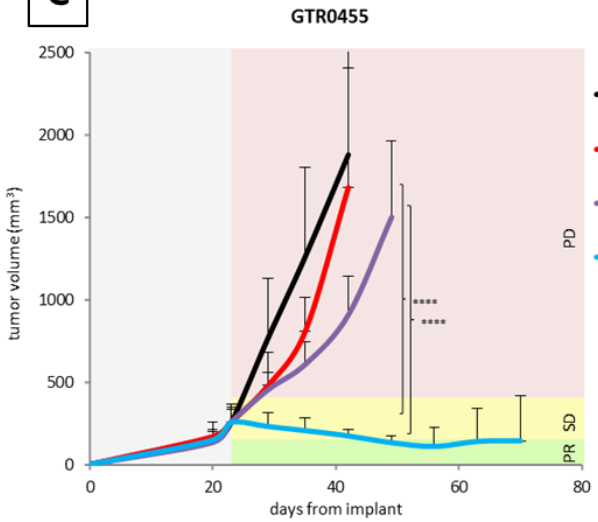

GTR0402
tumor volume (mm3)
days from implant
VEHICLE
TRASTUZUMAB
TRASTUZUMAB + LAPATINIB
TRASTUZUMAB + PERTUZUMAB
PD
SD
PR
****
****


GTR0402_METS
tumor volume (mm3)
days from implant
VEHICLE
TRASTUZUMAB
TRASTUZUMAB + LAPATINIB
TRASTUZUMAB + PERTUZUMAB
PD
SD
PR
****
****

B


GTR0402_METS
tumor volume (mm3)
days from implant
PD
SD
PR
TRASTUZUMAB
TRASTUZUMAB->DS-8201a

C


GTR0455
tumor volume (mm³)
days from implant
VEHICLE
TRASTUZUMAB
LAPATINIB
TRASTUZUMAB+ LAPATINIB
PD
SD
PR
****
****

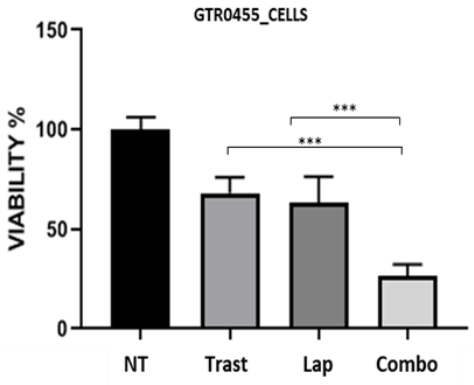

GTR0455_CELLS
VIABILITY %
***
***
NT
Trast
Lap
Combo

Figure 22: PDX models recapitulate patients' response to Trastuzumab. **A**. Tumor growth curves in mice cohorts derived from the GTR0402 tumor (left) or from the GTR0402 metastasis (right), treated with vehicle, Trastuzumab or the combos. **B**. Tumor growth curves of mice cohorts derived from GTR00402_METS, treated with trastuzumab (N=3) or with trastuzumab for 20 days and then with DS-8201a for 40 more days (N=3). Red arrow indicates start of trastuzumab treatment; blue arrow indicates start of DS-8201a treatment. Grey background: growth of the tumors before treatment start. **C** left panel. Tumor growth curves in mice cohorts derived from GTR0455 tumor, treated with vehicle, Trastuzumab, Lapatinib or the combo. Grey background: growth of the tumors before treatment start. The response in mice has been evaluated using RECIST 1.1-like criteria, as in Fig. 2. N = 6 mice; data are represented as mean + SD; ****p < 0.0001. Two-way ANOVA followed by Bonferroni multiple comparisons test has been used. **C** right panel. Cell viability assay performed on GTR0455 PDX-derived cells, upon treatment for 6 days with the indicated drugs (Trastuzumab 10µg/ml; Lapatinib 25nM).

PDX0455 (80 HER2 gene copies, was derived from a biopsy of a tumor showing primary resistance to Trastuzumab-containing treatment. Genomic analysis of the primary tumor and of the derived GTR0455 PDX model revealed, in addition to the 80 HER2 copies, the presence of an activating HER2 mutation (p.S310Y (188)) at the allelic frequency of 95%. The PDX was serially passaged in mice until six tumor-bearing animals were produced per experimental group. Xenografts were randomized into 4 cohorts and treated with (1) vehicle; (2) Trastuzumab; (3) Lapatinib; (4) Trastuzumab plus Lapatinib. In accordance with the clinical history of the donor patient, Trastuzumab-treated GTR0455 mice were resistant to treatment and underwent disease progression. No response was observed in Lapatinib-treated mice but the combination trastuzumab plus Lapatinib resulted in a strong reduction of tumor volume (figure 22C, left part). In vitro experiments performed in PDX-derived cells (which maintained HER2 amplification and mutation), exhibited poor susceptibility to either Trastuzumab or Lapatinib used as single-agents, but strong inhibition when used in combination (figure 22C, right part). Overall, our results show that the PDX models, in spite of the tumor heterogeneity, closely mirror the patient behavior and thus represent an invaluable tool to test new therapeutic approaches.

13. A NON-CELL AUTONOMOUS MECHANISM SUSTAINS ADAPTIVE SECONDARY RESISTANCE TO HER2 INHIBITION

As above shown, prolonged treatment of the GTR0233 PDX with anti-HER2 compounds in monotherapy resulted in tumor relapse (figure 23A). The genomic analysis of resistant tumors did not show any putative genomic alterations likely sustaining resistance to HER2 inhibition (data not shown). We thus investigated the onset of "adaptive" resistance sustained by activation of other receptor tyrosine kinases which could vicariate for HER2 activation. Our laboratory has recently shown that TKIs can induce non-cell-autonomous adaptive resistance to MET and EGFR targeted therapies through the secretion by Cancer-Associated Fibroblasts (CAFs) of the MET ligand, hepatocyte growth factor (HGF) (189). We thus wondered if this could be true also for HER2. Immunohistochemistry analyses showed increased phosphorylation of the MET receptor in lapatinib-resistant GTR0233 tumors compared to the matching sensitive ones (figure 23B). In situ hybridization with a mouse HGF RNA probe revealed that stroma of resistant tumor produced significantly more HGF than sensitive ones (figure 23C, D). Then, we isolated and grew in culture CAFs, both from wild type (sensitive) and resistant tumors. PCR analysis performed on CAF mRNA (figure 23E) and ELISA assay (figure 23F) conducted on culture supernatants showed that CAFs obtained from resistant tumors produced higher amount of HGF compared to wild type CAFs. To prove that stromal HGF-induced MET activation does sustain resistance, we performed an *in vivo* experiment co- treating resistant tumors—either few days after implant or when the tumors reached a volume of 250 mm^3—with both Lapatinib and Crizotinib (a dual MET/ALK inhibitor). As displayed in figure 23G, we observed that dual MET/HER2 inhibition prevented and overcame resistance in the above-mentioned settings, respectively. These results identify HGF stromal production as a new mechanism sustaining acquired resistance to HER2 inhibition.

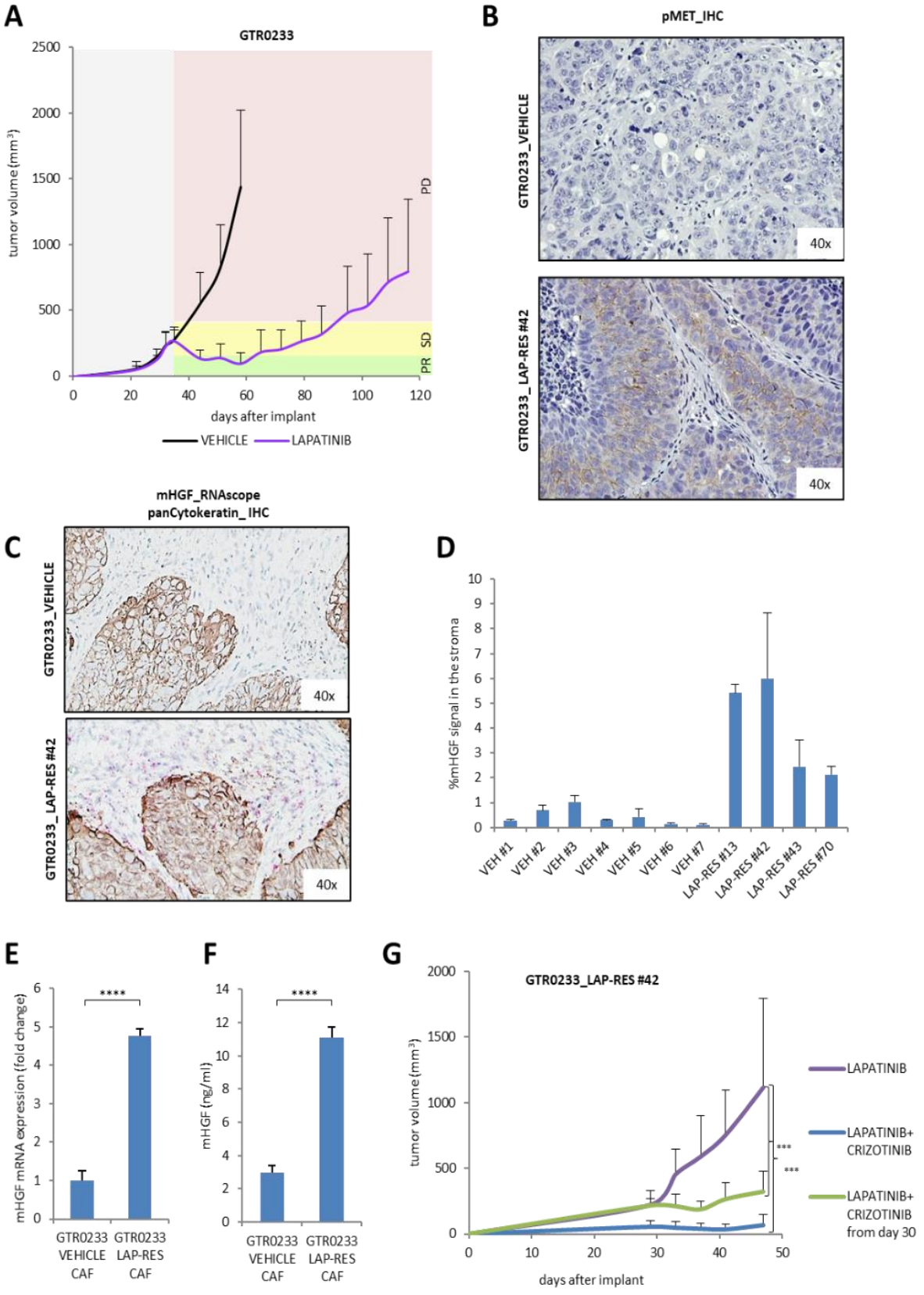

A
GTR0233
tumor volume (mm³)
2500
2000
1500
1000
500
0
0
20
40
60
80
100
120
days after implant
PD
SD
PR
VEHICLE
LAPATINIB
B
pMET_IHC
GTR0233_VEHICLE
40x
GTR0233_LAP-RES #42
40x
C
mHGF_RNAscope
panCytokeratin_IHC
GTR0233_VEHICLE
40x
GTR0233_LAP-RES #42
40x
D
%mHGF signal in the stroma
10
9
8
7
6
5
4
3
2
1
0
VEH #1
VEH #2
VEH #3
VEH #4
VEH #5
VEH #6
VEH #7
LAP-RES #13
LAP-RES #42
LAP-RES #43
LAP-RES #70
E
mHGF mRNA expression (fold change)
6
5
4
3
2
1
0
****
GTR0233 VEHICLE CAF
GTR0233 LAP-RES CAF
F
mHGF (ng/ml)
14
12
10
8
6
4
2
0
****
GTR0233 VEHICLE CAF
GTR0233 LAP-RES CAF
G
GTR0233_LAP-RES #42
tumor volume (mm³)
2000
1500
1000
500
0
0
10
20
30
40
50
days after implant
***
***
LAPATINIB
LAPATINIB+ CRIZOTINIB
LAPATINIB+ CRIZOTINIB from day 30

Figure 23: Identification of a non-cell autonomous, HGF-dependent mechanism of resistance to HER2 inhibition. **A.** Generation of a Lapatinib-resistant tumor. PDX GTR0233 has underwent prolonged treatment with Lapatinib, until resistance onset. Grey background: growth of the tumors before treatment start. The response in mice has been evaluated using RECIST 1.1-like criteria. **B**. IHC (pMET staining) of tumor slices obtained from the vehicle-treated (upper panel) and the Lapatinib-resistant tumor (lower panel). **C.** In situ hybridization with a murine-specific HGF probe (pink dots) of tumor slices obtained from the vehicle-treated (upper panel) and the Lapatinib-resistant tumor (lower panel). Slices have been also stained with panCytokeratin IHC to highlight tumor cells; **D**. Quantification of the mHGF mRNA signal in the stroma of tumors of either vehicle- treated or Lapatinib-resistant tumors. **E**. qRealTime PCR analysis of mouse HGF (mHGF) mRNA levels in cancer-associated fibroblasts (CAFs) derived from GTR0233 PDX untreated (vehicle) or resistant to Lapatinib (LAP-RES). **F.** Elisa assay quantifying the concentration of mouse HGF (mHGF) in the conditioned media of CAFs derived from GTR0233 PDX untreated (vehicle) or resistant to Lapatinib (LAP-RES). **G** .Tumor growth curves of mice cohorts derived from the GTR0233 patient (LAP_resistant #42), treated with the HER2 inhibitor Lapatinib, alone or in combination with the MET inhibitor Crizotinib, either few days after implant or when the tumors reached a volume of 250 mm^3. N = 5 mice for each model; data are represented as mean + SD; ***p < 0.001; Two-way ANOVA followed by Bonferroni multiple comparisons test has been used on GTR0455 PDX-derived cells, upon treatment for 6 days with the indicated drugs (Trastuzumab 10µg/ml; Lapatinib 25nM).

14. INDUCTION OF TRASTUZUMAB RESISTANCE IN SENSITIVE CELLS BY PRIMARY RESISTANT CELL-DERIVED EXTRACELLULAR VESICLES *IN VITRO* AND *IN VIVO*

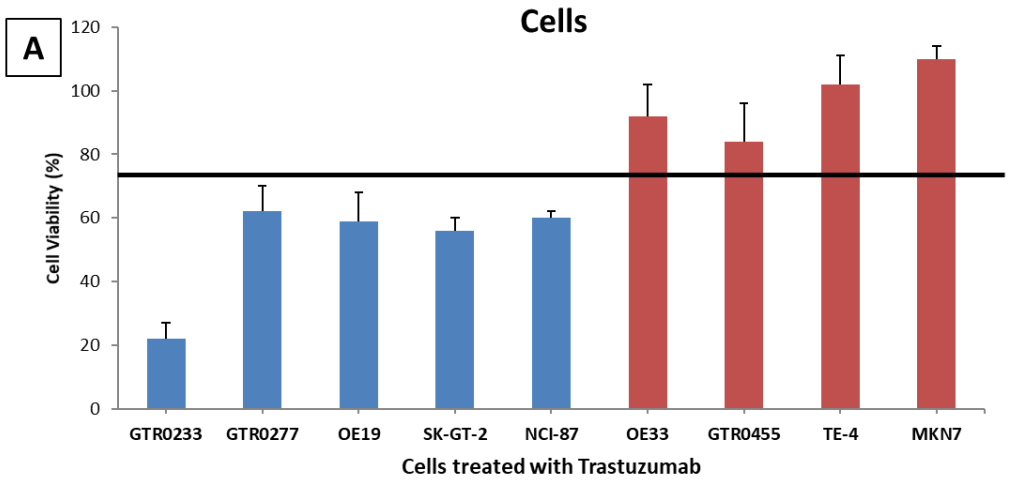
Although HER2-targeted therapy has been found to improve short-term outcome in HER2-positive GC, the success of such therapy achieved in HER2-positive breast cancer has not been effectively reproduced in patients with GC, highlighting the importance of understanding the mechanisms of resistance to HER2-targeted therapy in GC. Possible causes of Trastuzumab resistance have been proposed, including tumor heterogeneity in HER2 positivity, loss of HER2 protein expression, the tumor microenvironment and bypass pathways (190) (191) (192).

Extracellular vesicles attract increasing interest, and the biological functions of EVs-mediated exchange of drug resistance information between homogeneous or heterogeneous cells have been explored. Recent studies assessing different types of cancers revealed that EVs induce drug resistance by exporting drugs from cells and delivering resistance signals as well as neutralizing

antibodies (193). EVs are also potentially important in gastric cancer, but few studies of drug resistance have focused specifically on monoclonal agents. Therefore, it is important to explore the mechanism by which EVs derived from gastric cancer cells regulate Trastuzumab sensitivity. In line with this idea, we tested nine primary and immortalized gastric cancer cell lines bearing ≥ 10 HER2 gene copies and therefore theoretically sensitive to Trastuzumab. We observed that in vitro only five cell lines (GTR0233, GTR0277, OE19, SK-GT-2, NCI-87) displayed a cell viability below 70% upon Trastuzumab treatment. Four lines (OE33, GTR0455, TE-4, MKN7), instead, showed primary drug resistance (Figure 24A).

Next, we isolated the extracellular vesicles from the supernatant of all these cells by size exclusion chromatography. Quantity and quality of the isolated EVs was analyzed by Nanoparticle Tracking Analysis and Western blot confirming a high-quality standard (*data not shown*).

To assess the effects of EVs derived from the Trastuzumab-sensitive or Trastuzumab-resistant gastric cancer cell lines, the Trastuzumab-sensitive cell line GTR0233 was cultured for 6 hours in the presence of around $1x10^5$ EVs/recipient. Then, the cells were treated with Trastuzumab for other 72 hours. As shown in figure 24B, GTR0233 cell viability in the presence of Trastuzumab was increased by culture in the presence of EVs derived from Trastuzumab-resistant cells but not from Trastuzumab-sensitive cells, indicating that EVs can transfer drug resistance in Trastuzumab-sensitive cells. Similar results were also obtained with the SK-GT-2 , another Trastuzumab-sensitive cell line (*data not shown*). Interestingly, EVs derived from the Trastuzumab resistant TE-4 cells were unable to transfer resistance. The reasons for this behavior are currently under investigation. Indeed, several possible mechanisms by which extracellular vesicles induce drug resistance have been hypothesized to explain the results obtained in other tumor contexts. Among them is the presence in the vesicles of miRNAs differential expressed between sensitive and resistant cells. Therefore, we will evaluate by miRSeq the content of the vesicles obtained from all the gastric lines.



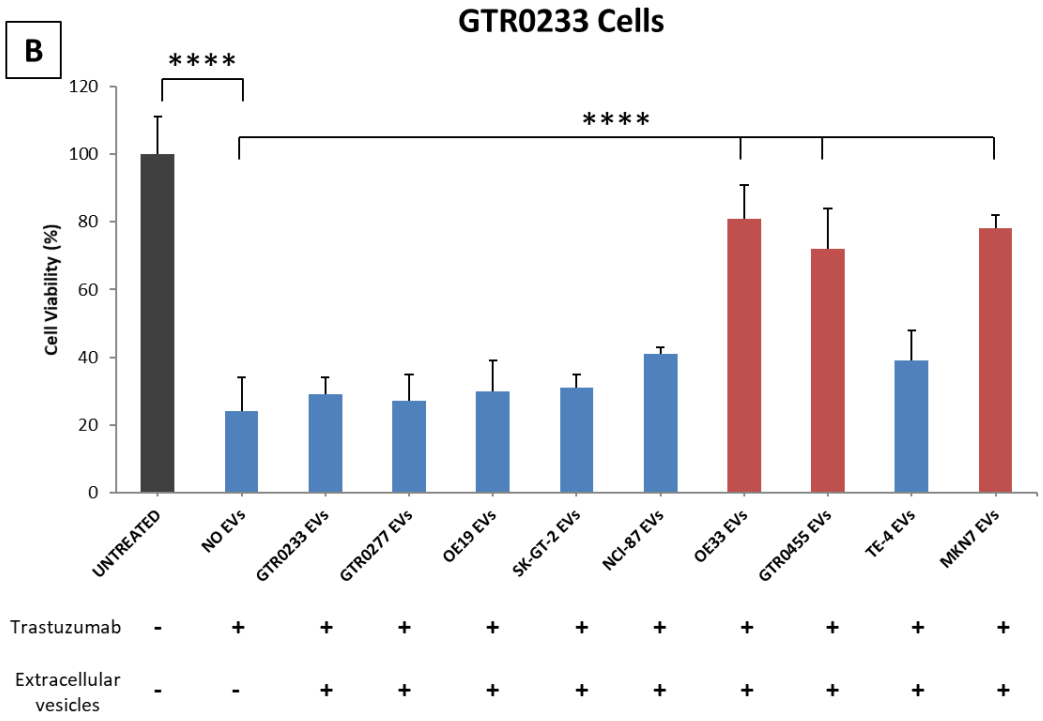


Figure 24: Induction of Trastuzumab resistance in sensitive cells *in vitro*. **A.** Cell viability assay performed on nine gastric cancer cell lines (primary and immortalized), upon treatment for 6 days with Trastuzumab (10 µg/ml). Responses (in blue) below the black bar are considered typical of Trastuzumab-sensitive cells. **B.** Cell viability assay performed on GTR0233: cells were cultured for 6 hours with EVs derived from the listed cell lines. Then, cells were treated with Trastuzumab (10 µg/ml) for another 72 hours. Data are represented as mean of biological triplicates + SD; ****p <0,0001. One-way ANOVA followed by Dunnett multiple comparisons test has been used.

Having obtained these first preliminary *in vitro* data, the next objective was to replicate the experiment *in vivo*, investigating whether the extracellular vesicles could induce resistance to Trastuzumab in tumor cells in a more complex system. Trastuzumab-sensitive cells GTR0233 were thus inoculated subcutaneously in mice. When the generated tumor formed a mass with an average volume of 150mm^3, mice were randomized into four experimental arms and treated twice 24 hours apart with: (i) EVs from OE33 (Trastuzumab-resistant cells), (ii) EVs from GTR0233 (parental sensitive cells), (iii) and (iv) PBS. Based on Nanosight evaluation, each injection contained approximately 1x10^{11} EVs (*data not shown*). 48 hours after the second EVs inoculation, mice were all treated weekly with Trastuzumab, except the vehicle. As shown in Figure 25, tumors treated with EVs from OE33 (Trastuzumab-resistant cells) and Trastuzumab had a PD similar to vehicle-treated tumors . In tumors treated with EVs derived from GTR0233 cells or PBS Trastuzumab led to SD, confirming the cell model sensitivity.

These findings confirmed, *in vitro* and *in vivo*, that EVs produced by HER2 positive cells resistant to Trastuzumab were able to render sensitive cells resistant to Trastuzumab.

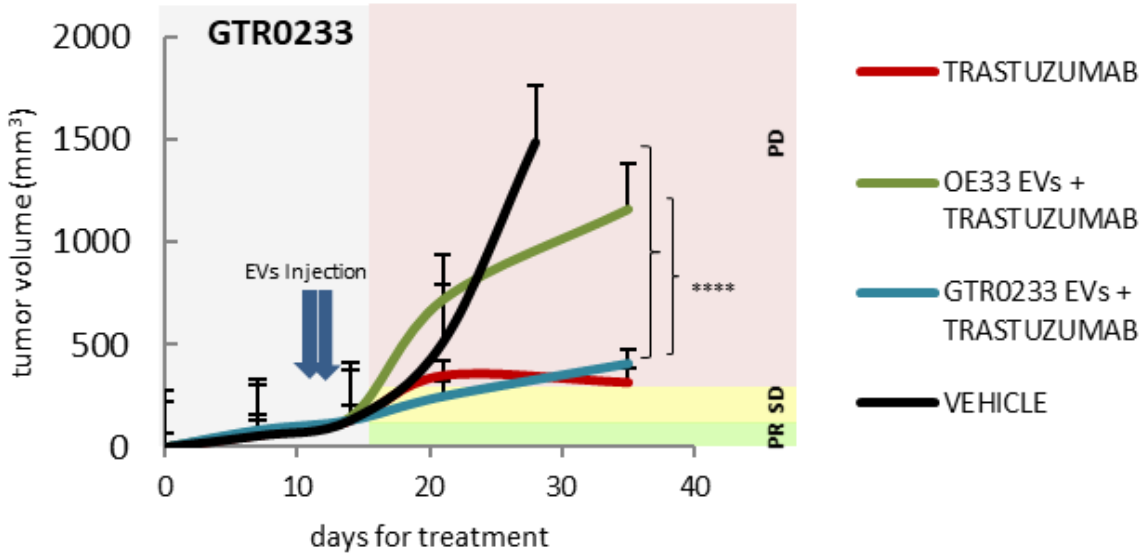


Figure 25: Induction of Trastuzumab resistance in sensitive cells *in vivo.* Tumor growth curves of mice cohorts derived from GTR0233 cells treated with extracellular vesicles (EVs derived from GTR0233 and OE33) and the HER2 inhibitor Trastuzumab. The blue arrows indicate the intratumoral inoculation of the EVs in two consecutive doses 24 hours apart. The treatment with the Trastuzumab started 48 hours after the second EVs injection. Grey background: growth of the tumors before drug treatment start. The response in mice has been evaluated using RECIST 1.1-like criteria, as in figure 17.

15. HER2 PHOSPHORYLATION IN EXTRACELLULAR VESICLES AS READ-OUT OF HER2 TARGETING

Extracellular vesicles are shed by cells under both normal and pathological conditions. They carry nucleic acids and proteins from their host cells that are indicative of pathophysiological conditions. Due to their presence and stability in most bodily fluids and resemblance of their contents to parental cells, EVs have a great potential to serve as a liquid biopsy tool for various diseases. In particular, cancer-derived EVs likely serve as biomarker for early detection of cancer as they carry the cargo reflective of genetic or signaling alterations in cancer cells of origin. Although HER2 is well known to be upregulated in tumor tissues, few studies have focused on circulatory HER2 released by extracellular vesicles (194).

Starting from the three available HER2 amplified PDX-derived primary cells (GTR0233, GTR0277, GTR0455), we first isolated by size exclusion chromatography the extracellular vesicles from the supernatant of the cells, previously cultured for 72 hours in a EVs free medium, and then determined the levels of the oncoprotein HER2. To identify a possible difference in the HER2 content, we also collected extracellular vesicles from two HER2 non-amplified models (GTR0245, GTR0498). Western blot analysis showed that only EVs derived from HER2 amplified cell models showed an increased presence of the HER2 protein, reflecting what happens in the cells of origin (Figure 24A). More importantly, HER2 phosphorylation was only detected in vesicles derived from amplified HER2 models. We also checked typical EVs marker proteins such as Alix and CD9, confirming the EVs successful isolation for all the cell lines.

These results demonstrate that GC extracellular vesicles contain not only the HER2 oncoprotein but also an activated HER2, which may play an important role as a biomarker and diagnostic factor.

To validate this biomarker as a response biomarker, we evaluated whether extracellular vesicles released by treated cells mirror drug-induced response of the cells themselves. As assessed previously (Figure 18A), by treating GTR0233 PDX-derived primary cells for 24 hours with Trastuzumab and Lapatinib (at a concentration corresponding to the IC50), the activated HER2 signal was downregulated. Collecting the supernatant after another 24 hours from the end of

the treatment and isolating the extracellular vesicles, we could observe that the amount of HER2 in the extracellular vesicles remained constant while the phosphorylation levels underwent a clear downregulation, perfectly mirroring what was observed in the cells (Figure 24B).
Together, these results show how the activated HER2 protein contained in EVs may parallel the active protein present in the cells. These preliminary data suggest the possibility of identifying in the blood of HER2 positive GC patients, not only the presence of HER2, but also whether the treatment with Trastuzumab is giving an effective pharmacological response.

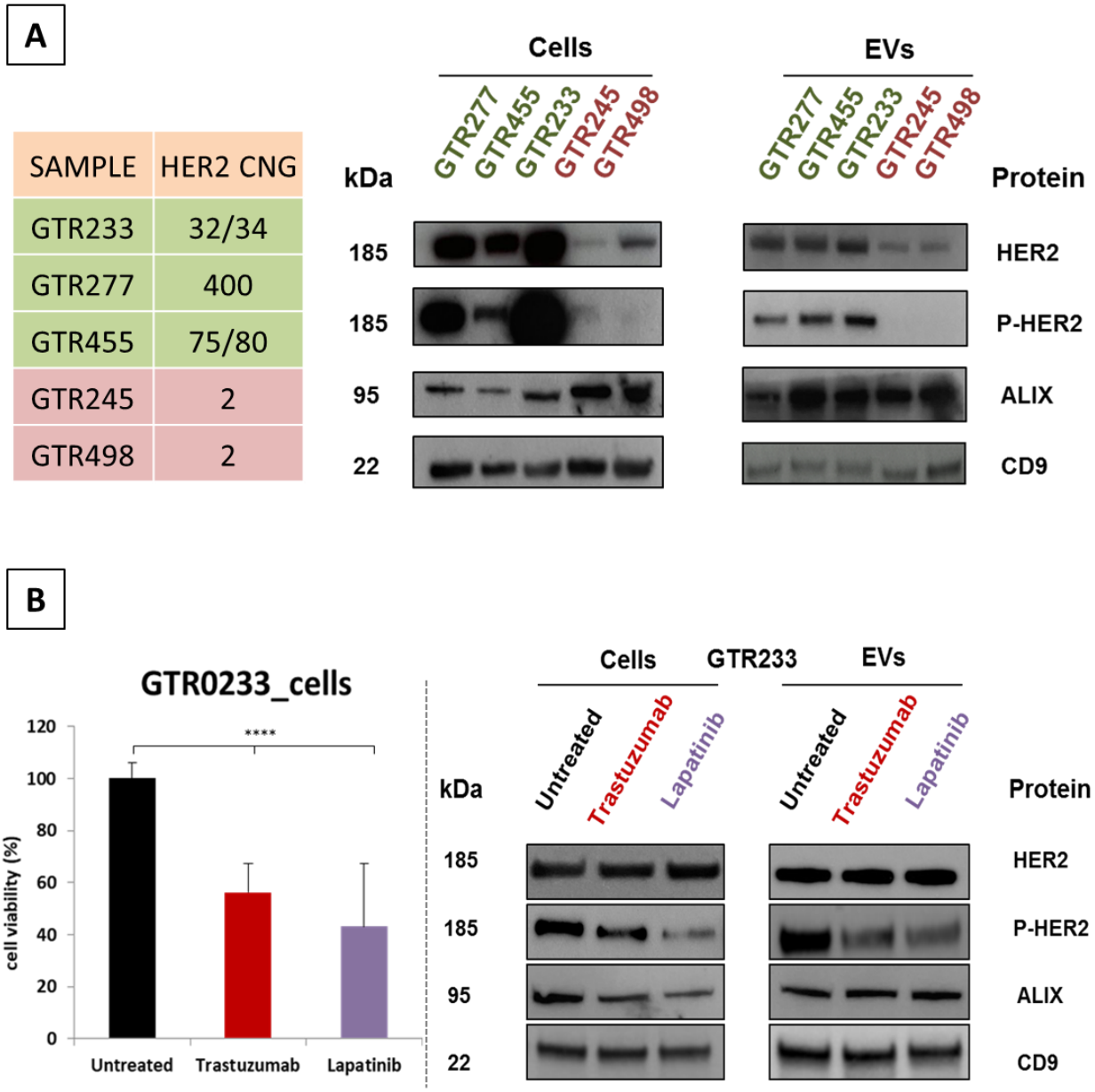


Figure 24: HER2 phosphorylation in EVs as read-out of HER2 activation in cancer cells. **A**. Western blot analyses performed on three PDX-derived cells with an "hyper" HER2 amplification (in green) and on two PDX-derived cells displaying two HER2 copies (in red), show the presence and the activation state of HER2 only in the HER2 amplified models. The presence of Alix and CD9, typical extracellular vesicles markers confirm the successful isolation of EVs through the size exclusion chromatography. **B** left panel. Cell viability assay performed on GTR0233 tumor-derived cells, upon treatment for 6 days with the indicated drugs at IC50 (Trastuzumab 0.15 µg/ml; Lapatinib 1 nM). **B** right panel. Western blot analyses showing the activation state of HER2 in GTR0233 tumor-derived cells treated for 24 hours with the indicated drugs (same doses used in the cell viability assays)and in the derived EVs collected 24 hours after the end of the treatment. Data are represented as mean of biological triplicates + SD; ****p <0,0001. One-way ANOVA followed by Dunnett multiple comparisons test has been used.

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DISCUSSION

Gastric cancer is still a leading cause of cancer-related death, representing a very challenging task in clinical oncology. Despite the significant advances in surgical techniques, improved diagnosis and development of new chemotherapeutic protocols, the clinical outcome of gastric cancer patients is still poor (15). To tackle this major disease there is an urgent need to better characterize this cancer from a molecular point of view and to identify new therapeutic targets. Human epidermal growth factor receptor 2 (HER2), is a key regulator of cell-survival, whose network of downstream signals leads to cell growth and proliferation. The frequency of HER2 amplification/overexpression in gastric and gastroesophageal cancer ranges from 4.4% to 53.4%, with a mean of 17.9% (195) (196).

Even if there is not a unanimous consensus, a large number of studies suggest that HER2 is a negative prognostic factor, with HER2-positive tumors showing a more aggressive biological behavior and higher frequency of recurrence (197) (198). With the approval of Trastuzumab for treatment of patients with advanced gastric cancer, the clinical demand for HER2 assessment has rapidly increased. However, HER2 testing in gastric cancer differs from testing in breast cancer because of inherent differences in tumor biology, intratumoral heterogeneity of HER2 expression and incomplete membrane staining that are commonly observed in gastric tumors (199). Moreover, only a fraction (<20%) of HER2-amplified patients benefit from Trastuzumab treatment, raising doubts about the true cost-effectiveness of this regimen in clinical practice. Notably, all recent trials with other anti-HER2 drugs have failed so far (66) (68).

One possible explanation for the poor activity of anti-HER2 drugs in HER2 positive gastric cancer patients is the lack of proper patient selection. Indeed, the goal of delivering the right drug to the right cancer patient requires a detailed understanding of how genomic alterations are linked to drug response. This allows the identification of positive and negative predictors of response for a critical and accurate selection of those patients that could benefit from therapy and to exclude resistant patients. Concerning positive predictors of response, it has recently been demonstrated that the level of HER2 gene amplification significantly predicts sensitivity to therapy, with a mean HER2/CEP17 ratio of 4.7 identified as the optimal cutoff value discriminating sensitive and refractory patients (178).

However, together with patients inclusion by means of positive predictors, personalized treatment requires negative predictors, to exclude primary resistant patients. In general, primary (or de novo) resistance involves the pre-existence of genetic lesions that drive the activation of collateral or downstream signaling pathways. Mechanisms of Trastuzumab resistance were mainly reported for breast cancer and comprise the activation of other HER family members (200), or of other membrane receptors (201), the alteration of the PI3K-mTOR pathway (202), the impairment of Trastuzumab ability to bind HER2 due to cleavage of HER2 extracellular domain (203) or to masking of the receptor by mucin-4 (204). In gastric cancer, very few mechanisms of resistance have been identified so far (205) (206) and validation of molecular markers as reliable negative response predictors is missing.

It is known that tyrosine kinase inhibitors (TKIs) are effective only in small sub fractions of patients, bearing tumors of a defined isotype. From a genetic point of view, each tumor type is, indeed, a collection of many relatively rare tumors, carrying different genetic alterations and thus responding to different molecular drugs. So, a prerequisite for the success of a targeted therapy is a precise molecular annotation of tumors, to properly select patients that could benefit from that therapy. However, in many cases, even a complete genetic map of the tumor is not sufficient to predict the response to a drug: if the genetic alteration is quantitative (e.g., oncogene amplification), it is often not clear which level of gene amplification leads to 'oncogene addiction', thus conferring drug sensitivity. Moreover, even a qualitative alteration (e.g., a mutation in a key oncogene) does not always reliably predict the response to a therapy. The paradigmatic example is the V600E mutation in BRAF that predicts response to Vemurafenib in 80% of melanoma patients, while it does not lead to a response to the same drug in colon cancer patients, as, in the latter tumor context, EGFR becomes activated upon BRAF inhibition (207). Moreover, the background of coexisting aberrations within cancer cells may also influence the outcome. Finally, even when a good molecular target has been identified for a specific tumor type, not all the inhibitors for that target are equally efficient in killing tumor cells, as demonstrated in colon cancer bearing HER2 amplification that -differently to what is observed in breast cancer- responds to the combination of Lapatinib plus Trastuzumab but is poorly sensitive to Trastuzumab alone (208).

All these issues severely restrain the clinical effectiveness of targeted therapies against gastric cancer.

The work of thesis aims to challenge these limits in order to optimize patient selection and improve treatment in gastric cancer patients. At the moment, the best preclinical model to validate targets and identify effective treatments is represented by Patient-Derived Xenografts (PDXs), which combine the flexibility of preclinical analysis with the informative value of population-based studies. Taking advantage of a proprietary, molecularly annotated colony of gastric cancer PDXs this work aimed to: (1) identify novel therapeutic strategies effective in Trastuzumab-resistant tumors; (2) provide a strong scientific basis for the rational selection of gastric cancer patients for anti-HER2 therapies through the identification of positive and negative predictors of response; (3) investigate novel mechanisms of resistance to Trastuzumab, in particular evaluating the role of extracellular vesicles in this context.

In order to identify the most effective therapeutic strategies in HER2 amplified tumors, we decided to use targeted drugs in the absence of any concomitant chemotherapy. We are also aware that in humans Trastuzumab activity is partly mediated by ADCC and that in NGS mice, due to the absence of the immune system, this effect is lacking. Our work, indeed, aimed to evaluate the direct and pure effect of HER2 pathway inhibition. In our experiments, thus, Trastuzumab was used as the "golden standard" of reference, to which we compared different drugs and drug combinations.

Other HER2 targeted drugs have been approved in HER2 amplified breast cancer; among them are Pertuzumab and Lapatinib. Pertuzumab is a humanized monoclonal antibody directed against the extracellular subdomain 2 of HER2 (recognizing a different epitope than Trastuzumab), which inhibits ligand-dependent heterodimerization of HER2 with other HER family members. This mechanism of action is different from that of Trastuzumab, which preferentially inhibits ligand-independent HER2 activation. The combo Pertuzumab plus Trastuzumab can thus (i) provide a more comprehensive blockade of HER signaling than either agent alone; (ii) promote a more effective endocytosis and degradation of the HER2 receptor, and consequently a lesser activation of the pathway. Lapatinib, used in the form of Lapatinib ditosylate, is a dual tyrosine kinase inhibitor that inhibits HER2 and EGFR pathways. The

literature has shown that TKIs are more effective on mutated receptors compared to antibodies. However, they do not induce receptor degradation but, on the contrary, increase the persistence of the receptors on the cell membrane. For this reason, as shown for example for HER2 positive colon cancer, the association with an antibody such as Trastuzumab (which induces receptor downregulation) can be advantageous.

Taking advantage of the unique opportunity provided by our wide platform of GC PDXs, we compared the efficacy of Trastuzumab monotherapy versus dual therapy (Trastuzumab + Pertuzumab or Lapatinib) in a particular subpopulation of HER2-positive cancers, bearing more than 8 HER2 gene copies, that we have called HER2 "hyper"-amplified.

Experience from Trastuzumab use in breast cancer has highlighted the importance of an appropriate HER2 evaluation to ensure accurate identification of patients eligible for anti-HER2 targeted therapies. Although the overexpression of the HER2 protein determined by IHC assays is a known predictive factor of response to Trastuzumab, the link between the level of HER2 amplification and the outcome in patients treated with Trastuzumab has been investigated only in breast cancer. Similarly, the relationship between the level of HER2 amplification and the outcome of HER2-positive gastric cancer treated with first-line chemotherapy with Trastuzumab remains unclear. Our aim was to determine whether the level of HER2 amplification significantly predicts increased response to therapy in GC, suggesting that only in a subpopulation of "hyper"-amplified HER2 tumors, the receptor plays a key role as an oncodriver and consequently the strongly enhanced activity of the combination of Trastuzumab plus Pertuzumab or Lapatinib, with their alternative and different mechanisms of action, may have a major impact

Our results show that, despite the high level of HER2 amplification and a homogeneous distribution of the overexpressed receptor in the tumor tissue, Trastuzumab elicited a PR only in 2 out of 8 PDXs, while dual therapy determined CR in 5 out of 8 cases (GTR0108; GTR0277; GTR0233; GTR0402; GTR0455). Most importantly, the deepness of response was significantly higher with the combos, leading to durable responses that in the two evaluated cases did not relapse even after drug withdrawal. Thanks to *in vitro* studies performed in the available PDX-derived cells, we showed that while Trastuzumab alone only slightly decreased the activation of

HER2 and its downstream targets, dual therapy was able to strongly impair or even abrogate it. A genetic rationale for the increased activity of Trastuzumab + Lapatinib or Pertuzumab was found in one case, GTR0277, displaying an activating mutation in HER3 (p.G284R). It has been hypothesized that this HER3 mutant acquires an untethered conformation of the extracellular domain relative to wild type and promotes oncogenic signaling in a HER2-dependent manner (183). Our results are in line with this hypothesis as the dual treatments were more active against HER2/HER3 heterodimers compared to Trastuzumab alone and were as efficient as the dual-HER2/ HER3 MoAbs. As a matter of fact, the presence of HER3 activating mutations may be a candidate genomic predictor of resistance to trastuzumab monotherapy and its role should be clinically validated in the frame of randomized clinical trials, such as JACOB (69).

All together these results suggest that the addition of either Pertuzumab or Lapatinib to Trastuzumab may be more effective than Trastuzumab alone in a subgroup of HER2 positive GC patients displaying high levels of HER2 amplification and in which HER2 may be regarded as the dominant driver of oncogene addiction. Our results are apparently discordant from the negative ones obtained in the JACOB study, which assessed the efficacy of first-line Pertuzumab versus placebo in combination with Trastuzumab and chemotherapy in HER2+ gastric or gastroesophageal junction adenocarcinomas. However, no post hoc molecular analyses have been performed up to date to identify the molecular profile of patients who benefitted from dual-HER2 blockade. Our data suggest that patients with a high degree of tumor HER2 amplification, coupled with lack of co-occurrent resistance alterations, are theoretically the optimal candidates for Pertuzumab-Trastuzumab combination strategies. Another possible reason of discrepancy can be linked to tumor heterogeneity. It is known that HER2 positivity in GC can be scattered in the tumor and the analysis of a single area does not necessarily reflect the majority of tumor cells. In our experience, we observed more that 90% positivity of cancer cells only in hyper amplified tumors, while in low amplified ones, the percentage of positive cells has often been quite low (although sufficient to score the tumor as HER2 3+ according to guidelines) and scattered inside the tumor.

We also noted that in our small cohort of xenopatients, the three cases harboring 8–10 HER2 gene copies did not respond to any HER2-targeted therapy. We may think that we should

consider the possibility to put a higher threshold to identify the truly HER2-dependent gastric carcinomas. All these considerations need a validation on a bigger number of cases and further strengthen the need of an accurate patient selection to optimally tailor patients' treatment.

In a patient who showed primary resistance to Trastuzumab-based treatment, we identified an activating HER2 mutation in the amplified HER2 gene (95% of allelic frequency both in the primary tumor and in the PDX). Thanks to the matching PDX (GTR0455), we showed its resistance to Trastuzumab or Lapatinib monotherapies, but response to Trastuzumab plus Lapatinib combination. Also in this case, experiments performed *in vitro* in PDX-derived cells confirmed the poor efficacy of monotherapies compared to dual therapy. This result shows that cases with concomitant presence of specific activating HER2 mutations can be targeted more efficiently with dual therapy.

In two PDX models, we tested the activity of antibody–drug conjugates already approved in breast cancer, such as trastuzumab–emantansine (T-DM1) and Trastuzumab deruxtecan.

T-DM1 showed efficacy in one of the two models (GTR0109). Interestingly, in the resistant model (GTR0031),only the new and more potent ADC trastuzumab deruxtecan was highly active. Intriguingly, the latter PDX model shows KRAS co-amplification (8 copies), a well-known biomarker of primary resistance to therapies targeting upstream receptors (74). Indeed, the co-occurrence of HER2 amplification and KRAS genomic alterations has been observed in 5% of GC patients in the TCGA database (21). In our experience, we have observed it in 10% of patients included in the AMNESIA study (4 out of 37 patients, all resistant to Trastuzumab) and in 10% of patients in our cohort (considering ≥ 4 HER2 and KRAS gene copies; 3% considering ≥ 8 HER2 and KRAS gene copies) (115). While the highly promising activity of Trastuzumab deruxtecan has been recently reported in a small cohort of patients with Trastuzumab-resistant HER2-amplified GC (72), we provide here the biological rationale for the use of HER2-directed ADCs to efficiently treat also those tumors displaying either primary or acquired Trastuzumab resistance. Notably, in the only two cases where we could compare the response to Trastuzumab in a patient and the corresponding PDX (namely GTR0402 and GTR0455), we observed a high similarity, further confirming the translational value of the obtained results.

In conclusion, this study suggests that the role of dual-HER2 blockade strategies should be re-assessed by randomized clinical trials aimed at focusing the enrolment of patients with HER2-positive GC to those with "hyper"-amplified status. Moreover, since the generation of evidence-based clinical data with novel targeted combinations is critically limited by the heterogeneity, multiplicity, and dynamic evolution of resistance mechanisms to Trastuzumab, as well as the undruggability of some of them (such as KRAS), the further clinical development of new ADCs, such as trastuzumab–deruxtecan, is highly warranted and should proceed in parallel with pre-clinical platforms. Translational cancer research will hopefully provide new tools to help clinicians to choose the best pharmacologic approach for a specific patient.

A newly explored mechanism by which tumors might counter the therapeutic effects of a targeted reagent involves their active release of extracellular vesicles into the extracellular environment, in malignant effusions, and also in tumor cell culture supernatants upon exocytic fusion of the multivesicular bodies with the plasma membrane (152). A broad spectrum of biological functions has been assigned to EVs, including the fact that they represent a pathway for expelling metabolites that could impair tumor cell survival and growth. Indeed, several reports have provided convincing evidence of a role for such nanovesicular structures in sustaining chemoresistance of tumor cells (209) (210).

Although extracellular vesicles were discovered 40 years ago, in the 1980s, there has been a dramatic progression in research intended to understand their biological significance and application to medicine. However, there are many details that still remain unknown, and new questions arise based on recent research. Several studies have reported the possible contribution of extracellular vesicles released by tumor cells to the generation of suppressive circuits that favor immune escape and promote cancer progression (171) (168). Our current data indicate a further detrimental role of these nanovesicles in cancer-carrying hosts by interfering with the bioactivity of therapeutic anticancer antibodies. EVs express discrete sets of tumor antigens, which include HER2 and EGFR, depending on the tumor type (194) (211). However, their biological role in HER2-overexpressing gastric tumors remain undefined. In the second part of the study, we have thus (i) characterized the constitutively secreted EVs from HER2 positive gastric carcinoma cells; (ii) analyzed HER2 status in EVs to monitor the effect of Trastuzumab

treatment of cells; (iii) investigated the role of EVs in inducing and sustaining resistance to Trastuzumab. Indeed, our analyses consistently provided evidence that HER2-positive EVs released *in vitro* from gastric cancer cells exhibited significant levels of activated HER2 and interfered with *in vitro* antiproliferative activity of trastuzumab.

Interestingly, primary cells provided by our wide platform exhibited different HER2 oncoprotein levels in isolated extracellular vesicles: amplified HER2 models had an evident presence of HER2 protein in EVs compared to non-amplified models, reflecting what happens in the cells. More importantly, HER2 phosphorylation was only detected in EVs obtained from amplified HER2 models. HER2 in GC extracellular vesicles therefore shows a possible important role as a biomarker and diagnostic factor. Furthermore, our data highlight how in EVs, HER2 can be considered a biomarker of response: the extracellular vesicles released by Trastuzumab-treated cells mirrored the response induced by the drug on the cells themselves, with a decrease of activated HER2 protein. These preliminary data suggest the possibility of analyzing in HER2 + patients' blood EVs not only the presence of HER2, but also its activation status, revealing if the pharmacological treatment is resulting in an effective response.

In addition to the aforementioned characteristics, recent studies have revealed that EVs play a key role in fostering the emergence of resistance to anticancer drugs through several mechanisms, including direct drug loading, expulsion and transfer of genes and proteins pro-survival, anti-apoptotic and associated with stemness (212). EVs are also potentially important in GC, but few drug resistance studies have focused specifically on monoclonal agents. We identified several primary and immortalized gastric cancer lines with ≥ 8 copies of the HER2 gene and tested their sensitivity to Trastuzumab: just over 50% of them demonstrated response to Trastuzumab *in vitro.* We showed that EVs derived from Trastuzumab-resistant cells (but not from Trastuzumab-sensitive cells) were able to decrease *in vitro* the response to Trastuzumab in Trastuzumab-sensitive cell lines, indicating that EVs can transfer drug resistance in Trastuzumab-sensitive cells. This finding has been confirmed also *in vivo* where treatment of Trastuzumab-sensitive tumors with EVs derived from Trastuzumab-resistant cells conferred resistance to Trastuzumab treatment.

These data raise potential concerns about the interference that HER2 overexpressing EVs may exert *in vivo* on the antitumor therapeutic activity of Trastuzumab. Indeed, cancer cells are known to produce EVs in an abundant and constitutive way (140) (161). Due to their specific physical structure and small size, such exosomes can easily recirculate in body fluids (213) (214) and they capture antibodies present in the bloodstream through specific binding according to their repertoire of surface proteins. Further experiments will need to be performed in physiologically relevant models to elucidate the EVs signaling network for clinical application. Therefore, more efforts still need to be devoted to better understand the roles and mechanisms of action of EVs in GC and to develop EVs-based clinical regimens for GC diagnosis, prognosis, and therapy. Indeed, there are some barriers to translate these findings in clinical practice. First, a standardization of the classification and extraction methods of EVs for different body liquids is urgently needed. More efficient methods with a low biofluid volume requirement and high purity and yield are the foundation of subsequent applications. Second, the identification of specific subtypes of EVs is mandatory, as different vesicles may exert diverse biological effects. Current methods to extract extracellular vesicles are too varied to confirm the purity of the product. Therefore, when attempting to use EVs in clinical testing, it is necessary to standardize protocols and methods.

In this work we have mainly investigated the role of EVs in the generation of resistance to targeted therapies and the possibility that they can play a role in the transfer of drug resistance. Since extracellular vesicles contain miRNAs, DNA, and proteins it is of paramount importance to identify which of these molecules is responsible for this biological ability of EVs. The development of appropriate identification techniques for these cargoes, along with the transfer mechanism will contribute to shed light on the development of drug resistance and assess predictive biomarkers for monitoring the efficacy of treatment regimens. Although clinical studies have established EVs as agents or target molecules, we are still far away from fully understanding the therapeutic aspect of these vesicles.

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PhD REPORT

Philosophiæ Doctor Activity Report

Publications

● Simona Corso, Marilisa Cargnelutti, Stefania Durando, Silvia Menegon, Maria Apicella, Cristina Migliore, Tania Capeloa, **Stefano Ughetto**, Claudio Isella, Enzo Medico, Andrea Bertotti, Francesco Sassi, Ivana Sarotto, Laura Casorzo, Alberto Pisacane, Monica Mangioni, Antonino Sottile, Maurizio Degiuli, Uberto Fumagalli, Giovanni Sgroi, Sarah Molfino, Giovanni De Manzoni, Riccardo Rosati, Michele De Simone, Daniele Marrelli, Luca Saragoni, Stefano Rausei, Giovanni Pallabazzer, Franco Roviello, Paola Cassoni, Anna Sapino, Adam Bass, Silvia Giordano. "Rituximab Treatment Prevents Lymphoma Onset in Gastric Cancer Patient-Derived Xenografts". Neoplasia. 2018/5/1. 20(5):443-455

● Simona Corso, Claudio Isella, Sara E Bellomo, Maria Apicella, Stefania Durando, Cristina Migliore, **Stefano Ughetto**, Laura D'Errico, Silvia Menegon, Daniel Moya-Rull, Marilisa Cargnelutti, Tania Capeloa, Daniela Conticelli, Jessica Giordano, Tiziana Venesio, Antonella Balsamo, Caterina Marchiò, Maurizio Degiuli, Rossella Reddavid, Uberto Fumagalli, Stefano De Pascale, Giovanni Sgroi, Emanuele Rausa, Gian Luca Baiocchi, Sarah Molfino, Filippo Pietrantonio, Federica Morano, Salvatore Siena, Andrea Sartore-Bianchi, Maria Bencivenga, Valentina Mengardo, Riccardo Rosati, Daniele Marrelli, Paolo Morgagni, Stefano Rausei, Giovanni Pallabazzer, Michele De Simone, Dario Ribero, Silvia Marsoni, Antonino Sottile, Enzo Medico, Paola Cassoni, Anna Sapino, Eirini Pectasides, Aaron R Thorner, Anwesha Nag, Samantha D Drinan, Bruce M Wollison, Adam J Bass, Silvia Giordano. "A Comprehensive PDX Gastric Cancer Collection Captures Cancer Cell–Intrinsic Transcriptional MSI Traits". Cancer Research. 2019/11/15. 79(22): 5884-5896

● Killian O'Brien, Koen Breyne, **Stefano Ughetto,** Louise C. Laurent & Xandra O. Breakefield. "RNA delivery by extracellular vesicles in mammalian cells and its applications". Nature Review Molecular Cell Biology. 2020/5/26. https://doi.org/10.1038/s41580-020-0251-y

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● **Stefano Ughetto**, Cristina Migliore, Filippo Pietrantonio, Maria Apicella, Annalisa Petrelli, Laura D'Errico, Stefania Durando, Daniel Moya-Rull, Sara E Bellomo, Sabrina Rizzolio, Tania Capelôa, Salvatore Ribisi, Maurizio Degiuli, Rossella Reddavid, Ida Rapa, Uberto Fumagalli, Stefano De Pascale, Dario Ribero, Carla Baronchelli, Giovanni Sgroi, Emanuele Rausa, Gian Luca Baiocchi, Sarah Molfino, Stefania Manenti, Maria Bencivenga, Michele Sacco, Claudia Castelli, Salvatore Siena, Andrea Sartore-Bianchi, Federica Tosi, Federica Morano, Alessandra Raimondi, Michele Prisciandaro, Annunziata Gloghini, Silvia Marsoni, Antonino Sottile, Ivana Sarotto, Anna Sapino, Caterina Marchiò,

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● Killian O’Brien, **Stefano Ughetto**, Xandra O. Breakefield. “Uptake, Functionality and Re-release of EV-encapsulated Cargo” Cell Report (under review).

●Koen Breyne, **Stefano Ughetto**, Rufino Ramos David Mahjoum, Shadi de Almeida Luis Pereira, Xandra O. Breakefield. "Supercharged Nanosized Biovesicles as a Trojan Horse for Efficient Delivery of Functional Cargo." ACS Nano (under review).

Conferences, meetings, and Awards

● EuroPDX Workshop: PDX models in clinical oncology and cancer precision medicine 3-5 October 2016
● ISEV2018 Annual Meeting 2-6 May 2018
● ASGCT 23RD Annual Meeting Tuesday, 12-15 May 2020 (Two Posters: > Extracellular Vesicles - A Trojan Horse for Therapeutic Agent Deliver > Extracellular Vesicles - A Tattletale for Rare Gene Editing Events).
● ISEV 2020 Annual Meeting 20-22 July 2020 (Poster: > Understanding Intracellular Fate of EV-delivered Content).
● ASEMV 2020 Annual meeting of the American Society for Exosomes and Microvesicles November 16 – 19, 2020 (***Poster Award***: Understanding intracellular fate of EV-delivery content)

Period of collaboration

From 01/04/2019 to 01/04/2020, visiting PhD student in the laboratory of Professor Xandra O. Breakefield Department of Neurology, Harvard Medical School and Geneticist (Neurology), Molecular Neurogenetics Unit, Massachusetts General Hospital.

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