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GASTRIC CANCER PDX/PDOX PRECLINICAL TRIALS: VALUABLE TOOLS TO DERIVE MOLECULAR INFORMATION FOR INNOVATIVE TARGETED THERAPIES AND STRATIFY PATIENTS FOR CLINICAL STUDIES.

THESIS' AUTHOR: **DOTT.SSA ROSSELLA REDDAVID**
SUPERVISOR: **PROF. MAURIZIO DEGIULI**

PHD PROGRAMME CO-ORDINATOR: **PROF PASQUALE PAGLIARO**
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INDEX

1. ABBREVIATIONS

GC	Gastric Cancer
EGC	Early gastric Cancer
cEGC	Clinically Early gastric Cancer
AGC	Advanced Gastric Cancer
FAGC	Far Advance Gastric Cancer
EBV	Epstein–Barr virus
LN	lymph node
EMR	endoscopic mucosal resection
ESD	endoscopic submucosal resection
EGD	Esophago gastro duodenoscopy
GIRCG	Italian Research Group for Gastric Cancer
CT	Chemotherapy
EBV	Epstein-Barr virus
MSI	Microsatellite instable
CIN	Chromosomal instable
GS	Genomically stable
MT	Molecular therapy
FDA	Food and Drug Administration
PDX	Patient-derived Xenograft
PDOX	Patient-derived Orthotopic Xenografts
AJCC	American Joint Committee on Cancer
RCT	Randomized Controlled Trial
EGFR	Epidermal Growth Factor Receptor
TKR	tyrosine kinase receptor
OS	overall survival
DFS	disease free survival
PFS	progression free survival
DM1	emtansine
ADC	antibody-drug conjugate
VEGF	Vascular Endothelial Growth Factor
ALK	Anaplastic lymphoma kinase
NSCLC	Non-small cell lung cancer
mTOR	Mammalian target of rapamycin
TOR	Target of rapamycin
TORC	TOR Complex
cMET	Hepatocyte Growth Factor Receptor
HGFR	Hepatocyte Growth Factor Receptor
SAE	Serious Adverse Effects
TCGA	Cancer Genome Atlas
MAb	Molecular Antibody

2. INTRODUCTION

2.1.Epidemiology, stage-related treatment and prognosis.

Gastric cancer is one of the most frequent malignancies. It represents the fifth most common tumor worldwide (5,6%) and the fourth leading cause of cancer-related death (7,7%) with 768793 deaths in 2020 (1). The majority of GCs are adenocarcinomas, which can be divided into intestinal and diffuse subtypes according to the Lauren Classification (2). A further classification, proposed by the World Health Organization, categorized GC into papillary, tubular, mucinous (colloid) and poorly cohesive carcinomas (3,4).

Most of GCs are strictly related to infectious agents, including the bacterium *Helicobacter pylori* and the Epstein–Barr virus (5). A small percentage of GCs are familiar and are characterized by mutations of E-cadherin (*CDH1*) (6) or of DNA mismatch repair genes (Lynch syndrome) (7).

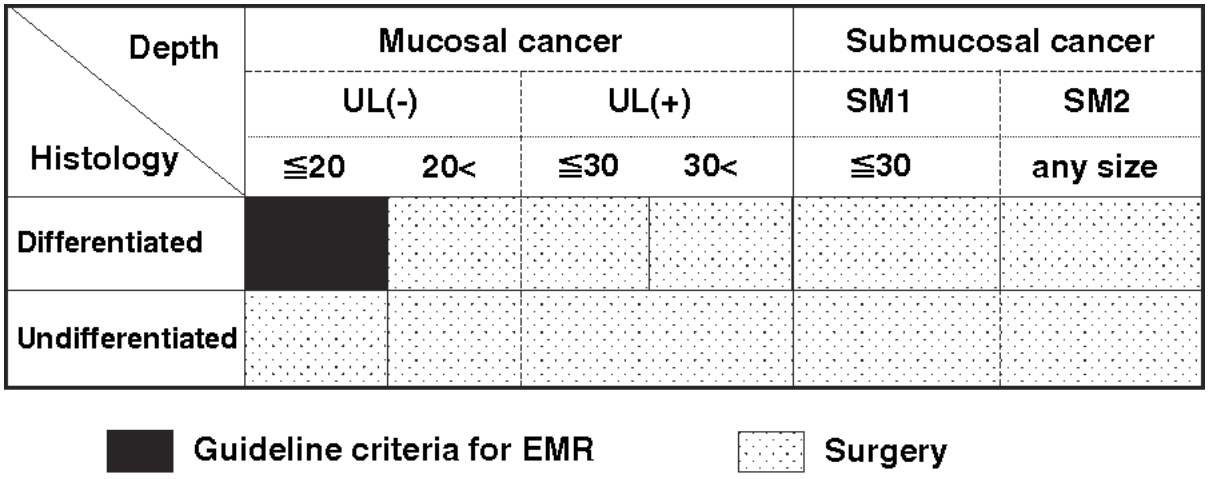
Based on their clinical stage at diagnosis GCs are divided in three main treatment groups: Early gastric Cancer, Advanced Gastric Cancer and Far Advanced Gastric Cancer or metastatic GC.

2.1.1. Early Gastric Cancer:

General guidelines for selecting EGC patients who are appropriate for curative endoscopic resection are primarily based upon the risk of lymph node metastasis as observed in previous surgical resections (8). Proper staging is therefore crucial for determining which patients are potential candidates for endoscopic treatment. Patients meeting Gotoda's established criteria can be safely submitted to EMR because they are expected to be free from LN metastases (9) (Fig 1a). Expanded criteria for endoscopic resection were implemented by high-volume centers from Eastern Asia. Nonetheless, to date, ESD still remains under evaluation and patients meeting these criteria should be considered only for treatment in experimental arms of controlled trials and restricted to referral centers (10) (Fig 1b). Nevertheless, the number of patients harboring an EGC and submitted to endoscopic treatment is increasing, considering the excellent long-term outcomes of EMR and ESD in case of EGC meeting the established or expanded criteria. These patients have a good prognosis with survival rates close to 100%. Many authors reported 5-year overall and disease specific survival rates of more than 92% and 99%, respectively, in patients submitted to endoscopic resection (11,12), whereas the 5-year cumulative incidence of recurrent gastric cancer is quite high, ranging from 2.9% to 14% (13,14). Even though a potential risk of distant metastasis after ESD remains because lymph node dissection is not performed in patients undergoing ESD, its effective incidence was reported to be extremely rare (15). However, as documented in 5- or 10-year long-

term follow-up data available in literature, there were some extragastric recurrences after curative ESD (16). Therefore, annual or biannual surveillance EGD together with abdominal computed tomography scan might be necessary for at least 5 years after curative endoscopic resection for early gastric cancer, regardless of the type of indication (established as well as expanded).

a



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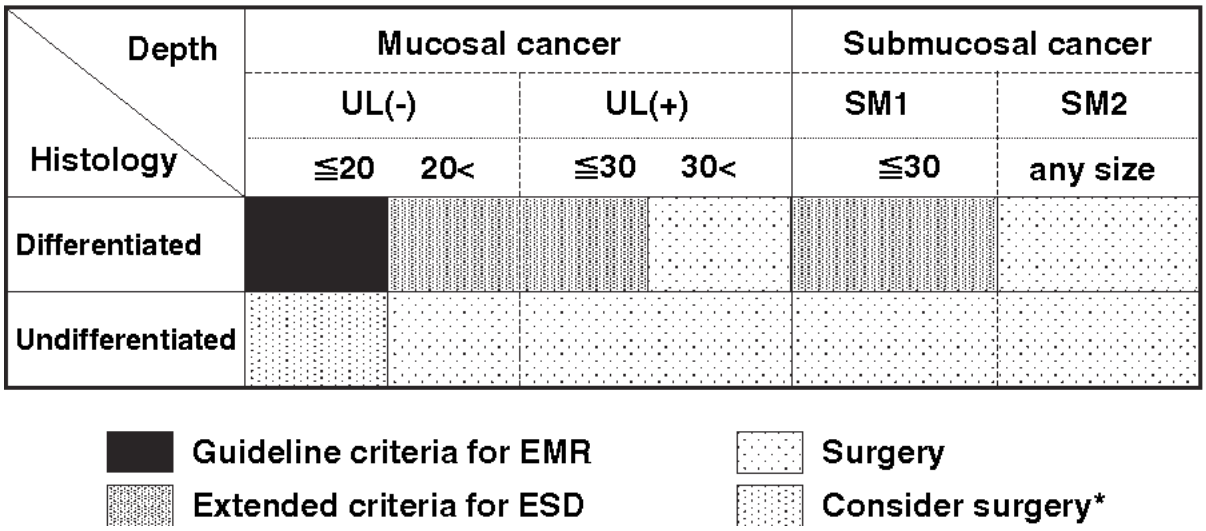

Figure 1. **a**. Guideline criteria for endoscopic resection in the endoscopic mucosal resection (*EMR*) era. Size is shown in mm. *UL*, ulcerative findings; *SM*, submucosal invasion. **b**. Proposed extended criteria for endoscopic resection in the endoscopic sub- mucosal dissection (*ESD*) era. *Asterisk*; although the possibility of metastasis is very low in this category of patients, surgery is considered because endoscopic en-bloc removal is sometimes difficult in undifferentiated-type tumors

Early gastric cancer patients who underwent endoscopic resection (EMR or ESD) and have risk factors for LN metastasis or who don't meet Gotoda criteria in light of histological data, should be submitted to surgery with at least D1 lymphadenectomy (8). Indeed, surgical treatment with adequate lymphadenectomy can offer a high probability of definitive cure as documented by 5- and 10-year cancer-related survival rates (98% and 95% respectively) reported in case of pT1N0 EGC treated with appropriate node dissection (17). Usually, a D1 or a D1plus lymph node dissection are considered as adequate dissections and a proper D2 lymphadenectomy is not required for EGC. Indeed, two large European randomized controlled studies comparing survival after D1 or D2 gastrectomy for gastric cancer didn't report any significant differences of 5-year disease specific survival for stage I patients (18,19).

Regarding the surgical approach, in this last decade many randomized controlled trials investigated the long-term outcomes of minimally invasive gastrectomy compared with open technique for EGC. In 2006, the KLASS-01 trial reported that overall and cancer-specific survival rates were comparable in patients receiving either laparoscopic or open distal gastrectomy (94.% and 97% respectively) (20). In 2020, the JCOG0912 study confirmed the non-inferiority of laparoscopic as compared to open distal gastrectomy for relapse-free survival in case of stage I gastric cancer, suggesting that laparoscopic distal gastrectomy should be considered as a standard treatment option for EGC when performed by experienced surgeons (21).

Despite the overall good prognosis of EGC, some subtypes show a significantly worse oncological outcome. An Italian prospective study with a large sample size documented that the risk of positive nodes is particularly high in diffuse-mixed type, an aggressive form of gastric cancer with special propensity to lymph node metastasis (22). Another EGC subtype which harbors a poor prognosis is the PenA Kodama type, which represents more than one fifth of these early tumors (23). Many studies reported that PenA histologic characteristic represents a negative prognostic factor, especially in N positive patients. Indeed, among node positive patients, non-PenA patients showed a plateau in terms of survival after 5 years while survival continued to lower and a plateau was reached only five years later in PenA patients (24,25). Finally in 2006 the Italian Research Group for Gastric Cancer retrospectively analyzed 652 cases of resected EGC and established that submucosal invasion, Lauren diffuse/mixed type, Kodama Pen A type and tumor size are significantly associated with an increased risk of lymph node metastases (17). For all these reasons, the GIRCG guidelines advice a D2 lymphadenectomy in cEGC not suitable for endoscopic treatment (26).

2.1.2. *Advanced Gastric Cancer*

In this last decade, evidence-based medicine and practical surgical experience moved towards a global agreement: at present, D2 procedure (Fig 2) is recommended as the standard surgical treatment for resectable AGC. This recommendation is fully accepted by the Japanese, Korean, German, British, and Italian medical and surgical oncology societies and by the European Society for Medical Oncology (ESMO) and the joint ESMO- ESSO (European Society of Surgical Oncology)- ESTRO (European Society of Radiotherapy and Oncology) guidelines. In addition, more recently, the NCCN Clinical Practice Guidelines in Oncology recommended the D1+ or modified D2 procedure also in the United States (27).

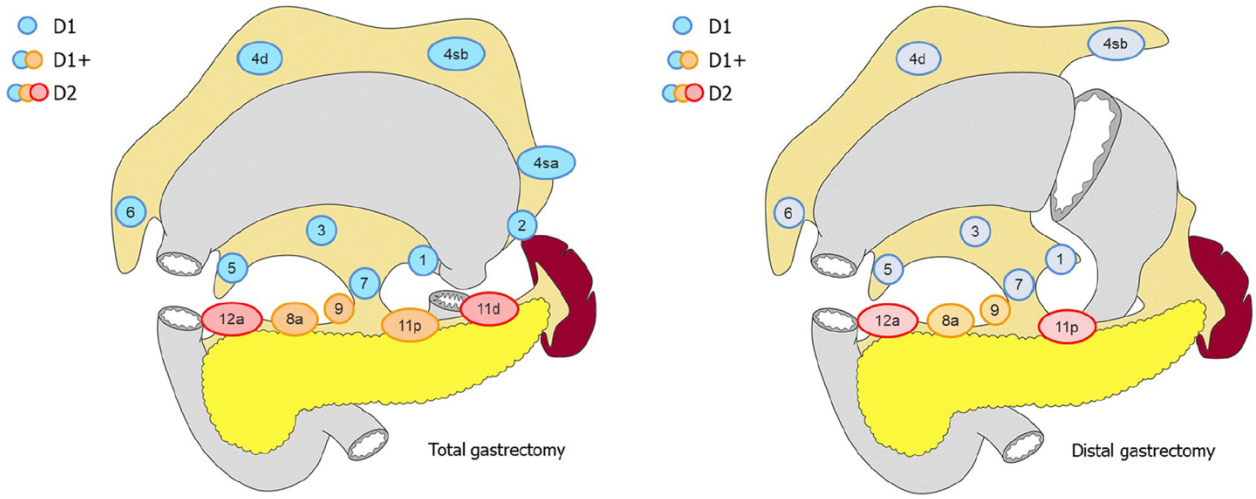

Figure 2. Lymph node dissection in **total gastrectomy** and in **distal gastrectomy**. Lymph node stations in blue need to be dissected in D1 dissection. In addition, lymph node stations in orange need to be dissected in D1+ dissection and lymph node stations in red as well in D2 dissection.

Survival outcomes of patients submitted to upfront surgery with adequate lymph node dissection for AGC are similar in both eastern and western countries, the 5-year OS for AJCC stage II and III ranging from 44% to 86% and from 22% to 64% respectively (18,19,28,29). To our knowledge, there is no reliable multicenter RCT showing a long-term outcome of laparoscopic gastrectomy for advanced gastric cancer until now, while several phase III trials investigating the non-inferiority of this procedure as compared to open gastrectomy in terms of long-term outcomes are ongoing (30–33).

In recent times a multimodal approach of GC has been recommended with the adoption of different neoadjuvant (preoperative or perioperative) treatment regimens; this was also included in several national guidelines of many western countries, particularly after the publication of the Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC) and of the Federation Nationale des Centres de Lutte contre le Cancer (FNCLCC)- Federation Francophone de Cancerologie Digestive (FFCD) randomized controlled trials. Unfortunately this was done without a strong evidence-based medicine (EBM)-related demonstration of a survival benefit as compared to controlled surgery alone with proper enlarged LN dissection in patients with proper stomach tumors (34). Nevertheless, a novel safe and effective regimen of neoadjuvant treatment, a docetaxel-based combination consisting of fluorouracil, leucovorin, oxaliplatin, and docetaxel (FLOT), has been recently introduced with a significant improvement of survival outcomes compared with previous pre- and peri-operative treatment schedule for patients with locally advanced, resectable gastric or gastro-esophageal junction adenocarcinomas. Indeed, Al-Batran reported 3- and 5-year OS rates of 57% and 45% respectively with the administration of FLOT regimen (35).

A recent retrospective study of GIRCG supports the hypothesis that “posterior” lymph node stations (8p,12p and 13) should be considered as regional nodes in case of advanced distal gastric cancer, and para-aortic nodes (12a2, 12b1) in case of advanced diffuse proximal tumors (36). Another study from GIRCG could document that D2 plus (previously called D3) LN dissection, including the systematic removal of posterior and para-aortic stations (8p, 12p, 13, 16a2 and 16b1) (Fig 3), could reverse the negative impact of diffuse histotype on locoregional recurrence. Therefore, D2 plus could be considered a valid therapeutic option in histotype-oriented tailored treatment of AGC (37).

Nr.	Definition
1	Right paracardial LNs, including those along the first branch of the ascending limb of the left gastric artery
2	Left paracardial LNs including those along the esophagocardiac branch of the left subphrenic artery
3a	Lesser curvature LNs along the branches of the left gastric artery
3b	Lesser curvature LNs along the 2nd branch and distal part of the right gastric artery
4sa	Left greater curvature LNs along the short gastric arteries (perigastric area)
4sb	Left greater curvature LNs along the left gastroepiploic artery (perigastric area)
4d	Rt. greater curvature LNs along the 2nd branch and distal part of the right gastroepiploic artery
5	Suprapyloric LNs along the 1st branch and proximal part of the right gastric artery
6	Infrapyloric LNs along the first branch and proximal part of the right gastroepiploic artery down to the confluence of the right gastroepiploic vein and the anterior superior pancreatoduodenal vein
7	LNs along the trunk of left gastric artery between its root and the origin of its ascending branch
8a	Anterosuperior LNs along the common hepatic artery
8p	Posterior LNs along the common hepatic artery
9	Coeliac artery
10	Splenic hilar LNs including those adjacent to the splenic artery distal to the pancreatic tail, and those on the roots of the short gastric arteries and those along the left gastroepiploic artery proximal to its 1st gastric branch
11p	Proximal splenic artery LNs from its origin to halfway between its origin and the pancreatic tail end
11d	Distal splenic artery LNs from halfway between its origin and the pancreatic tail end to the end of the pancreatic tail
12a	Hepatoduodenal ligament LNs along the proper hepatic artery, in the caudal half between the confluence of the right and left hepatic ducts and the upper border of the pancreas
12b	Hepatoduodenal ligament LNs along the bile duct, in the caudal half between the confluence of the right and left hepatic ducts and the upper border of the pancreas
12p	Hepatoduodenal ligament LNs along the portal vein in the caudal half between the confluence of the right and left hepatic ducts and the upper border of the pancreas
13	LNs on the posterior surface of the pancreatic head cranial to the duodenal papilla
14v	LNs along the superior mesenteric vein
15	LNs along the middle colic vessels
16a1	Paraaortic LNs in the diaphragmatic aortic hiatus
16a2	Paraaortic LNs between the upper margin of the origin of the celiac artery and the lower border of the left renal vein
16b1	Paraaortic LNs between the lower border of the left renal vein and the upper border of the origin of the inferior mesenteric artery
16b2	Paraaortic LNs between the upper border of the origin of the inferior mesenteric artery and the aortic bifurcation
17	LNs on the anterior surface of the pancreatic head beneath the pancreatic sheath
18	LNs along the inferior border of the pancreatic body
19	Infradiaphragmatic LNs predominantly along the subphrenic artery
20	Paraesophageal LNs in the diaphragmatic esophageal hiatus
110	Paraesophageal LNs in the lower thorax
111	Supradiaphragmatic LNs separate from the esophagus
112	Posterior mediastinal LNs separate from the esophagus and the esophageal hiatus

Figure 3. Anatomical definitions of lymph node stations. LNs: Lymph nodes.

2.1.3. Far Advanced (Metastatic) Gastric Cancer

Chemotherapy remains the main therapeutic approach for stage IV GC. Indeed, despite recent developments in chemotherapy, the median survival time of these patients remains very low, ranging from 13 to 16 months (38). Japanese treatment guidelines state that the role of gastrectomy is unclear in patients with stage IV GC. Actually, reduction surgery aims to prolong survival or to delay the onset of symptoms by reducing tumor volume (8). An international cooperative randomized controlled trial showed that stage IV patients can benefit from surgery in terms of survival only when this is radical (39). In 2017 Yamaguchi retrospectively collected 77 stage IV GC patients submitted to conversion surgery and the median survival was 41.3 for R0 patients while it was 21.2 months for R1-2 (40).

Several studies reported that conversion surgery for unresectable stage III or stage IV GC is associated with longer survival than chemotherapy alone. Most recent studies on stage III/IV unresectable patients undergoing conversion surgery reported survivals ranging from 37 to 56 months (40,41). The Italian Group of Gastric Cancer Research recently documented that conversion gastrectomy is a treatment option for selected patients with stage IV GC. The main prognostic factor for these patients is the presence of more than one type of extra-gastric metastatic involvement and a radical procedure is significantly associated with a reduced risk of recurrence (42).

The algorithms of standard treatment based on the stage of GC at its diagnosis are reported in Appendix 1.

Hence, in the last decade several perioperative and postoperative regimens of conventional CT have been investigated, and neoadjuvant treatment has been recommended as mandatory in several national guidelines, but the prognosis of stage III and IV GC remains poor (34,43–45). This has been one of the reasons for the researchers to investigate further aspects of GC.

2.2.Molecular classification

In 2014, the Cancer Genome Atlas Research Network paved the way for a new molecular classification of GC and documented the existence of four major genomic subtypes: Epstein-Barr virus (EBV; 9%), microsatellite instable (MSI; 22%), chromosomal instable (CIN; 50%), and genomically stable (GS; 20%) (46) (Fig 4).

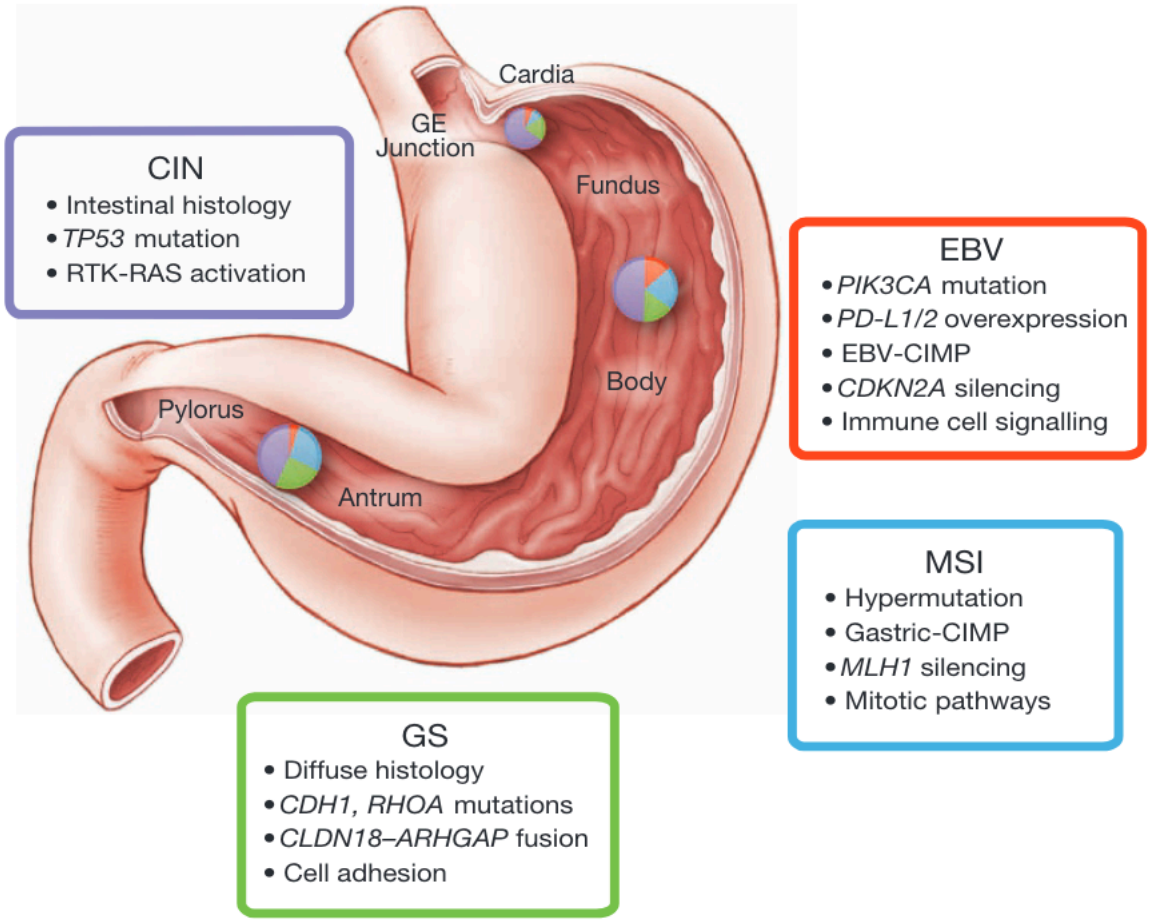

Figure 4. Key features of gastric cancer subtypes. This figure lists some of the salient features associated with each of the four molecular subtypes of gastric cancer. Distribution of molecular subtypes in tumours obtained from distinct regions of the stomach is represented by inset charts.

The identification of these subtypes and the related signaling pathways provided a roadmap for GC patient stratification and promising strategies for targeted therapies.

2.2.1. Epidermal Growth Factor Receptor

EGFRs include four types of TKRs (HER1/EGFR, HER2, HER3, HER4) located on the cell surface. They play an important role, conveying messages to manage cell growth and differentiation.

a. Anti-EGFR

Many authors have demonstrated that approximately 30% of GCs show EGFR overexpression (47,48). Two main monoclonal antibodies (cetuximab and panitumumab) that inhibit EGFR activity by binding its extracellular domain have been identified. Moreover, cetuximab can stimulate the activity of the immune system against tumor cells (49). Unfortunately, the heterogeneity of GC seems to affect the efficacy of cetuximab in most of these patients (50). Gefitinib and erlotinib, two tyrosine kinase inhibitors, can also inactivate EGFR by binding its intracellular domain and blocking its kinase activity (51). Unfortunately, phase II trials have shown that these therapies have limited efficacy in molecularly unselected patients (52,53). Recently, we and others (54,55) have identified a subpopulation of GC patients presenting a high level of EGFR amplification, which is responsive to anti-EGFR drugs. Mechanisms of resistance to EGFR-targeted drugs, such as TKR activation, KRAS mutation/amplification, and TSC2 inactivation, have also been identified (55).

b. Anti HER2

Several authors have shown HER2 gene amplification (and the consequent overexpression of its receptor) in many types of tumors (56). The HER2 gene is a proto-oncogene located on chromosome 17q21. The first approved HER2 inhibitor is the monoclonal antibody trastuzumab. In 2010, the ToGa trial documented the superiority of trastuzumab in combination with conventional chemotherapy compared with chemotherapy alone in terms of OS and DFS for patients with HER2-amplified AGC (57). Nevertheless, only a few patients with GC (less than 20%) gain a real advantage from trastuzumab.

In the past decade, several other anti-HER2 agents (such as Lapatinib, a dual kinase inhibitor that inhibits EGFR and HER2, and Pertuzumab, an anti-HER2 monoclonal antibody that prevents heterodimerization) have been tested for GC treatment and failed (58,59). The efficacy of the combination of trastuzumab and pertuzumab has been investigated in the JACOB trial (60). Despite the suggestion of treatment activity (a trend towards therapeutic activity for increasing PFS and the proportion of patients who achieved an objective response), adding pertuzumab to trastuzumab and chemotherapy did not significantly improve OS in patients with HER2-positive GC vs. placebo.

T-DM1 is an antibody–drug conjugate generated by the conjugation of trastuzumab and DM1, a tubulin inhibitor (61). The action of this drug is characterized by two phases: first, the ADC binds the extracellular domain of HER2; it is subsequently transferred intracellularly, releasing DM1 that proceeds to block microtubule polymerization. The GATSBY trial, a randomized, open-label, adaptive, phase II/III study investigating the efficacy of T-DM1 compared to taxane in patients with previously treated, HER2-positive AGC, didn't evidence a significant benefit of T-DM1 (62). Trastuzumab deruxtecan (DS-8201) is an antibody–drug conjugate consisting of trastuzumab, a cleavable linker, and a cytotoxic topoisomerase I inhibitor. An openlabel, randomized, phase II trial performed on HER2+ GC patients evaluated trastuzumab deruxtecan vs. chemotherapy and showed that treatment with trastuzumab deruxtecan led to significant improvements in response and OS compared with standard therapies (63).

2.2.2. Vascular Endothelial Growth Factor

VEGFs are proteins promoting blood vessel formation. Four types of VEGF (VEGF-A, VEGF-B, VEGF-C, and VEGF-D) have been identified, with three types of corresponding receptors (VEGFR-1, VEGFR-2, and VEGFR-3). Several studies have reported the fundamental role of these signaling proteins in new blood vessel formation and cancer cell proliferation (64). Furthermore, VEGF expression has been found in approximately 40% of GC (65). Bevacizumab is an anti-VEGF-A monoclonal antibody that prevents the binding with its receptor (66). The efficacy of this monoclonal antibody has been widely documented in several solid tumor treatments (67–69) but bevacizumab is still under investigation for its benefit in GC. Some phase II/III trials proved its efficacy in association with conventional chemotherapy in AGC, while others did not report any clear benefits (70,71). Furthermore, Shah et al. reported improved oncologic outcomes only in Caucasian patients but not in Asian patients, suggesting that the VEGF-A pathway in GC could be different among races (72).

Many trials have investigated the efficacy of VEGF TKR inhibitors (sunitinib and sorafenib), but no phase III trial has shown any survival benefits (73,74). Finally, a monoclonal antibody blocking VEGFR-2 was successfully introduced for advanced solid malignancy treatment in 2010 (ramucirumab) (75). A significant improvement in survival outcomes in patients with AGC submitted to second-line therapy with ramucirumab alone or in combination with paclitaxel was documented in two main phase III trials and led to the approval of this drug in GC (76,77). Interestingly, these two trials also highlighted significant differences in the VEGF-A pathway between Asian and non-Asian patients.

2.2.3. Mammalian Target of Rapamycin

mTOR is a serine/threonine protein kinase identified in mammalian cells with a leading role in controlling mechanisms of cell growth and proliferation. Human cancers can be characterized by hyperactivity or inactivity of the mTOR pathway, which plays a crucial role in maintaining tumor-modified phenotypes (78). In 2008, Cejka et al. (79) demonstrated *in vitro* the efficacy of everolimus (RAD001) in inhibiting mTOR complex 1 (mTORC1, mTOR combined with the adaptor protein raptor) with consequent blockage of HIF-1 and VEGF. The authors concluded that everolimus, through the inhibition of mTORC1 in GC cells, could affect cancer proliferation and generate central tumor necrosis. Moreover, everolimus antitumor action is amplified by its association with metronomic cyclophosphamide.

2.2.4. Hepatocyte Growth Factor Receptor

HGFR, also known as c-MET, is a proto-oncogenic receptor tyrosine kinase that, after binding to hepatocyte growth factor, induces cell migration and proliferation, promotes mitosis, and inhibits apoptosis. C-MET overexpression and gene amplification are related to a poor prognosis (80,81).

*Okamoto et al. in 2012 stated that crizotinib (a tyrosine kinase inhibitor of c-MET and of the anaplastic lymphoma kinase) "has pronounced effects on signal transduction and survival in gastric cancer cells with MET amplification" (82). Phase II/III trials to evaluate crizotinib efficacy and safety in GC are ongoing.

Another promising agent targeting the HGF-cMET complex is rilotumumab. This human monoclonal antibody impairs the c-MET signaling pathway by binding to and inactivating its ligand HGF (83). Clinical trials of this drug in GC (including two phase III trials) were halted due to a significant increase in mortality in the experimental arm (rilotumumab in combination with conventional chemotherapy) in one of these trials, but new investigations have begun.

Finally, the METGastric, a phase III trial of onartuzumab (a humanized monoclonal antibody that binds to the extracellular receptor of c-MET) plus standard first-line chemotherapy for HER2, was recently conducted in MET+ advanced GC (84). This phase III trial was stopped early because of negative results reported in a concomitant Phase II study that concluded: "The addition of onartuzumab to mFOLFOX6 in gastric cancer did not improve efficacy in an unselected population or in a MET immunohistochemistry-positive population" (85,86).

Targeted therapies and oncogenic pathways in gastric cancer are detailed in Figure 5.

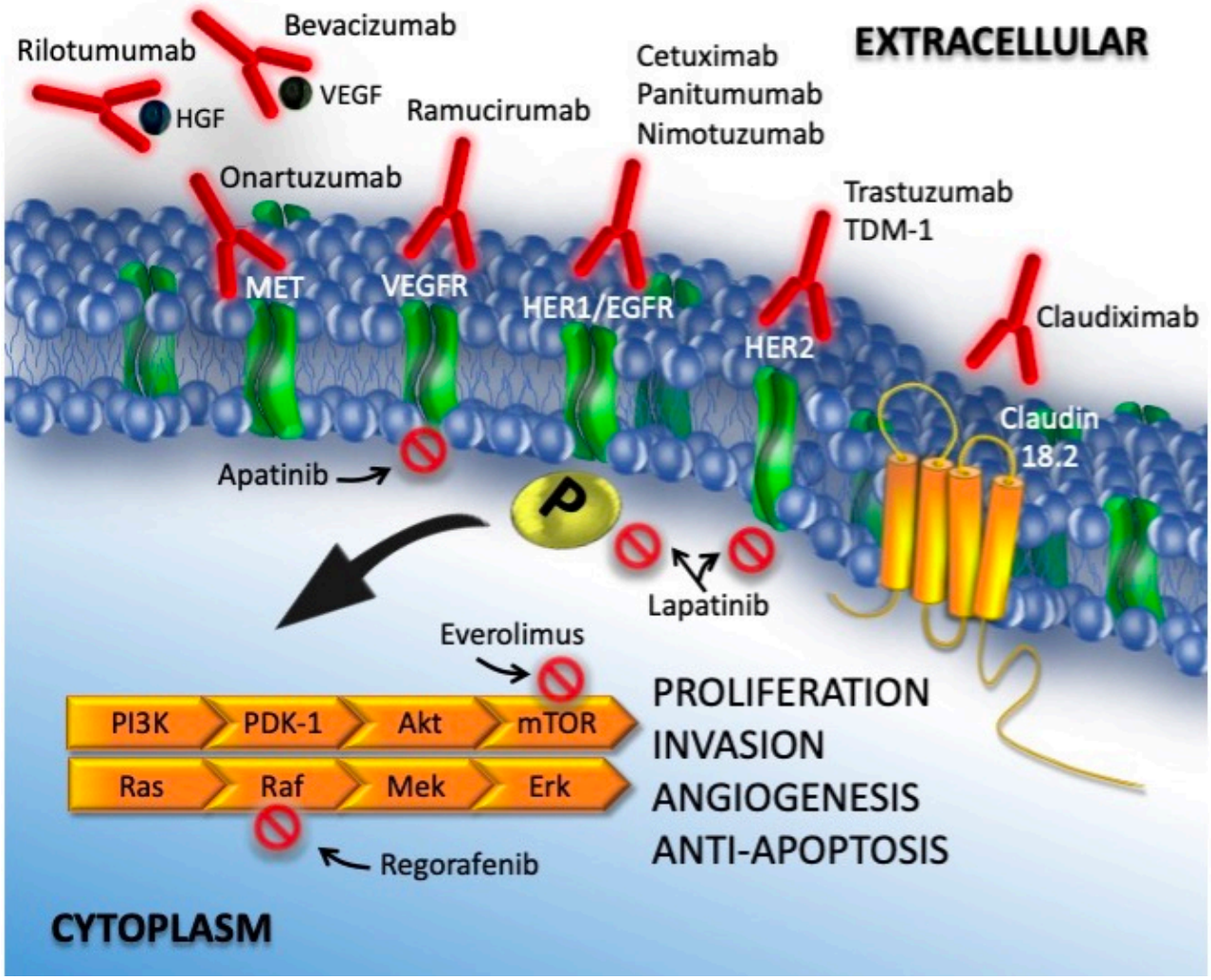

Figure 5. Targeted therapy and oncogenic pathways in gastric cancer. Activation of ERK-AMP KINASE. Ligand binding to a growth factor receptor activates the small GTP-binding RAS protein which interacts with RAF protein kinase. RAF phosphorylates and activates MEK (MAP kinase or ERK kinase) which then activates ERK (extracellular signal-regulated kinase) by phosphorylation of tyrosine and threonine residues. Activated ERK translocates into the nucleus where it phosphorylates the Elk-1 transcription. PI3K/AKT/MTOR pathway. PI3K/AKT/MTOR signaling constitutes an important pathway that consist of two steps: phosphatidylinositol 3-kinase (PI3K) and its downstream molecule serine/threonine protein kinase B (PKB; also known as AKT). The PI3/AKT/mTOR pathway is stimulated by RTK and cytokine receptor activation. Tyrosine residues are then phosphorylated and provide anchor sites for PI3K translocation to the membrane thus participating in the transduction of various extracellular matrix molecules and cytokines, including mTOR, a serine/threonine protein kinase, is a member of the PI3K-associated kinase protein family.

2.3.Preclinical trials

To explore the molecular mechanisms supporting tumour growth and its responsiveness to medical treatment, animal models are very helpful. Actually, the best preclinical model to validate targets and positive/negative predictors of response to therapy is represented by Patient-derived xenografts (PDXs), an experimental model that retains the principal histologic and genetic characteristics of the donor tumour, is predictive of clinical outcome, and is a valuable tool for personalized medicine decisions (87). This model summarizes many of the disease hallmarks of cancer patients and is increasingly being applied to investigate existing and new drug therapies, tumour growth, and mechanisms of drug resistance. PDXs are usually generated by transplantation of human tumour tissue or cells into seriously immunodeficient mouse host strains. Tumours that successfully engraft are further passaged to generate cohorts of tumour-bearing mice for experimental studies. PDX models are generated and used by researchers in academic, clinical, and pharmaceutical industry settings as well as specialized commercial organizations (88).

Human subcutaneous tumour xenografts, grown in immunodeficient nude mice, morphologically, biologically, and biochemically closely resemble the original tumours. The major problem of PDXs generated by subcutaneous implant is that the transplanted tumours are located in an abnormal microenvironment. Most subcutaneously implanted tumours are encircled by a pseudocapsule; having little chance to spread to the surrounding tissues, they very rarely metastasize (89–91), regardless of their origin from highly aggressive tumours (92). However, human tumours implanted orthotopically (that is, in the organs of origin of the tumour) in nude mice (PDOX) show increased metastatic capability (92–95). Therefore, human gastrointestinal tumours, orthotopically transplanted in athymic mice, can contribute to enhance our knowledge of cancer spreading and metastasis.

Recently, the technique of orthotopic xenograft has been ameliorated, from the “sewing” method to the “adhering” method (96–98). The progress made in the operative technique strongly decreases the procedure duration and improves animals’ morbidity and mortality. Therefore, PDOX were recently introduced in GC preclinical research to better recapitulate the original cancer background (93).

2.4.Clinical trials

Trastuzumab was the first molecular therapy approved by the FDA and European Union for AGC; it was subsequently introduced as the standard of care for patients with locally or FAGC displaying HER2 overexpression/amplification (57). In 2014, FDA also approved the use of ramucirumab as monotherapy or in combination with paclitaxel for advanced and metastatic GC (99). To date, only these two MAbs (in addition to the antibody-drug conjugate trastuzumab deruxtecan) have been approved, although many other molecular targets have been identified in recent years. Indeed, the majority of phase III trials investigating novel molecular agents failed to demonstrate their efficacy, mostly due to inaccurate patient selection (particularly concerning driver gene amplification and copy number) and the lack of preclinical models supporting proof of concepts followed by structured trials (100) (Tab 1). In addition, several studies have shown different escape mechanisms of cancer cells that could shorten the duration of or even nullify the response to targeted therapies. Furthermore, a strict relationship between c-MET amplification copy number and the response grade to anti-MET therapies have been documented.

Author, Trial, year	EXP arm	CTR arm	Molecular target	Results
Waddell, REAL-3(101), **2009**	EOC + Panitumumab	EOC	EGFR	
Ohtsu, AVAGAST(102), **2012**	XP + Bevacizumab	XP + Placebo	VEGF	
Sahin, FAST(103), **2012**	EOX + Claudiximab	EOX	Claudin 18.2	
Pavlakis, INTEGRATE(104), **2012**	Regorafenib	Placebo	VEGF, RET, RAF	
ENRICH (NCT01813253), 2013	Irinotecan + Nimotuzumab	Irinotecan	EGFR	
Hecht, LOGiC(105), **2013**	XELOX + Lapatinib	XELOX + Placebo	HER2	
Satoh, JapicCTI-090849(106), **2014**	Irinotecan + Nimotuzumab	Irinotecan	EGFR	
Wilke, RAINBOW(77), **2014**	Paclitaxel + ramucirumab	Paclitaxel + placebo	VEGFR2	
Fuchs, REGARD (107), **2014**	Ramucirumab	Placebo	VEGFR2	
Bang, ToGA(57), **2014**	FP/XP + trastuzumab	FP/XP	HER2	
Satoh, TyTAN(58), **2014**	PTX + Lapatinib	PTX	HER2	
Ohtsu, GRANITE-1(108), **2015**	Everolimus	Placebo	mTOR	
Lordick, EXPAND (109), **2016**	XP + Cetuximab	XP	EGFR	
Li(110), **2016**	Apatinib	Placebo	VEGFR-2	
Shah(85), **2017, METGastric**	Onartuzumab+ FOLFOX6	Placebo+ FOLFOX6	MET	
Thuss-Patience (62), **2017, GATSBY**	Trastuzumab+ emtasine	Taxane	HER-2	
Catenacci, 2017(111), **RILOMET-1**	Rilotumumab + ECX	placebo + ECX	HGF	
Cunningham(112), **2017, UK Medical Research Council**	Bevacizumab+ ECX	ECX	VEGF	
Fuchs(113), **2019, RAINFALL**	Ramucirumab + Fluoropyrimidine+cisplatin	Placebo+ Fluoropyrimidine+cisplatin	VEGFR-2	
Lorenzen (114), **2020, RADPAC**	Paclitaxel+ Everolimus	Placebo+Paclitaxel	mTOR	
Shah(115), **2020 GAMMA-1**	Andecaliximab+ mFOLFOX6	Placebo+ mFOLFOX6	MMP9	

Table 1. Results of phase II and III trials. This table summarizes recent phase II and III RCTs investigating novel molecular agents' survival outcomes. Unfortunately, most of these trials did not show any overall and progression free survival advantages as compared to conventional chemotherapy (red dot). Positive and partially positive studies have been pointed out with green and orange dot, respectively.
Nr: number; pts: patients; EXP: experimental; CTR: control; XELOX: capecitabine and oxaliplatin; EOC/EOX: epirubicin + oxaliplatin + capecitabine, XP: capecitabine and Cisplatin, FP: 5-fluorouracil and cisplatin, PTX: paclitaxel, FOLFOX6: fluorouracil leucovorin oxaliplatin; ECX: Epirubicin cispaltin and capecitabine; mFOLFOX6: modified oxaliplatin, leucovorin, and fluorouracil; MMP9: Matrix metalloproteinases 9.

positive study partially positive study negative study.

3. AIM OF THE STUDY

The aim of the project was to generate a wide PDX and PDOX esophago-gastric cancer platform including all the histologic and molecular types diagnosed in human patients

Our definitive purpose was to identify and validate molecular targets and positive/negative predictors of response to therapy in preclinical studies employing patients' derived cellular coltures, organoids and xenografts.

4. Results

4.1. Paper n°1

In this analysis, we found that every 3DL surgical resection cost around 250 euros/procedure more than 2DL but every operation rescue around 320 Euros in terms of operative time save.
The difference of price between the two equipment is about 48000 Euros. The maintenance service is the same but the spare parts and repair labor could be more expensive for 3DL than for 2DL.
In conclusion, the 3DL is a promising technology that appear not to need a great starting investment and not to weight on the cost of single procedure. That could mean a sustainability even in small centers.
We present the results of a monocentric use of 3DL, the extensive use has to be better valuated to assesses the advantage in the treatment of tumor of right colon and money saving
New technologies.

Abstract 21
PRIMARY THYROID LEIOMYOSARCOMA: A CASE REPORT

G.L. Canu [1], E. Erdas [1], J.S. Bulla [2], G. Baghino [2], C. Salaris [1], S. Mariotti [2], P.G. Calò [1]. [1]*Department of Surgical Sciences, University of Cagliari, Cagliari, Italy;* [2]*Endocrinology Unit, University Hospital and University of Cagliari, Cagliari, Italy*

Background: Primary thyroid leiomyosarcoma (LMS) is a very rare tumor, with only a few cases described in literature. LMS is supposed to arise from thyroid capsule, particularly from smooth muscle cells of the vessels. Preoperative diagnosis is challenging because of the similarity with other malignant tumors. Surgery is the primary treatment modality, while benefits of chemotherapy and radiotherapy are controversial. The prognosis is poor with an estimated 1-year survival rate of 5–10%.
Case report: A 47-year-old man was referred to our unit for a rapidly growing mass in the neck and dysphagia. Physical examination revealed a 5–6 cm firm mass in the right anterior cervical region, fixed to superficial and deep tissue layers. The patient was clinically and biochemically euthyroid and thyroid autoantibodies were undetectable. Thyroid ultrasonography showed a heterogeneous hypodense mass (60 x 39 x 33 mm) of the right thyroid lobe, with peri- and intralesional vascular flow and deflection of the trachea. There was no evidence of lymphadenopathy and distant metastases on CT scan. Fine-needle aspiration cytology was diagnostic of undifferentiated malignancy (Class 6 Bethesda). At surgery, a hard and irregular mass of the right thyroid lobe, infiltrating the esophageal wall, was found. Consequently, a total thyroidectomy with partial esophagectomy was performed. Postoperative course was uneventful and the patient was discharged 7 days after surgery in good conditions. Istological and Immunohistochemical evaluations (marked reactivity with Vimentin, Desmin, Smooth Muscle Actin and Specific Muscle Actin, Ki67 positive in 40% of neoplastic cells) allowed the conclusive diagnosis of primary thyroid high-grade LMS. At two-months follow-up, a local recurrence was detected at 18F-FDG PET/CT and the patient was referred to the Oncological Unit to start chemotherapy (Adriamycin + Ifosfamide). The patient is still alive 3 months after surgery.
Conclusion: A rare case of thyroid LMS has been described. The final histological diagnosis required immunochemistry investigation to exclude other more frequent aggressive thyroid tumors. LMS must be taken into account in patients presenting with rapidly growing neck masses. At the moment, there is no standard therapy and the prognosis is poor.
Head-Neck.

Abstract 39
PERITONEAL METASTASES FROM ENDOMETRIAL CANCER TREATED WITH CYTOREDUCTIVE SURGERY (CRS) COMBINED WITH HYPERTHERMIC INTRAPERITONEAL CHEMOTHERAPY (HIPEC): A REPORT ON 33 PATIENTS

T. Cornali [1], J. Spiliotis [2], D. Biacchi [1], N. Kopanakis [2], B.M. Sollazzoi [1], A. Christopoulou [2], A. Impagnatiello [1], P. Sammartino [1]. [1]*Sapienza University of Rome, Rome, Italy;* [2]*Metaxa Cancer Hospital, Athens, Greece*

To learn more about peritoneal metastases from endometrial cancer (PMEC) treated with cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) we collected data prospectively from two tertiary referral centres experienced in treating peritoneal surface malignancies (PSM).
In a retrospective series including all patients treated with CRS and HIPEC for PMEC at the Metaxa Cancer Hospital (Greece) and at the Department of Surgery P. Valdoni (Italy) from November 2002 to April 2016, we analysed the main demographic, clinical and outcome data. We included patients with PMEC younger than 75 years with Eastern Cooperative Oncology Group performance status 0–2, resectable disease and informed written consent and excluded those with extra-abdominal disease, other malignancies except breast cancer, unresectable disease or patients unfit for the procedure. Follow up data were completed on December 31 2016.
Thirty-three consecutive patients (mean age 59 years, range 42–73) were treated, 5 for primary disease and 28 for recurrence. Preoperative management included in 78.8% patients systemic chemotherapy (mean 1.5 lines, range 0–3). The mean peritoneal cancer index (PCI) was 15 (range 3–35) and surgery obtained complete cytoreduction in 60.6% of the patients. HIPEC was done with closed technique with a solution of cisplatin at a dose of 75 mg/m^2 for 60 minutes. The major morbidity rate was 21% and the operative mortality 3%. With a median follow-up of 48.1 months, the median overall survival for the entire cohort was 31.1 months while the median progression-free survival was 26 months. Multivariate regression analysis identified the completeness of cytoreduction score (CC-S) as the only factor factors worsening long-term survival.
Conclusions: In this series, that to our knowledge is the largest so far of peritoneal metastases from endometrial origin treated with CRS and HIPEC, the only significant prognostic factor for OS is the CC-S.
Furthermore, our outcome data show that in patients with PMEC CRS and HIPEC can be performed with acceptable morbidity and mortality rates and that such an increased survival compared to median survival reported with other treatments encourage further larger studies including and randomized trials.
Peritoneal carcinomatosis.

Abstract 64
VALIDATION OF A MODEL OF ORTHOTOPIC TRANSPLANTATION OF HUMAN GASTRIC CANCER IN NOD SCID MICE FOR GENERATION OF A GASTRO-ESOPHAGEAL PATIENT-DERIVED XENOGRAFT (PDX) PLATFORM TO IMPROVE THERAPEUTIC OUTCOME

R. Reddavid [1], S. Menegon [2], S. Corso [2], S. Giordano [2], M. Degiuli [1]. [1] *San Luigi University Hospital, Torino, Italy;* [2] *Istituto di Candiolo IRCC, Torino, Italy*

Gastric cancer is the third leading cause of cancer mortality worldwide. Surgery is the only curative treatment strategy; conventional chemotherapy has shown limited efficacy and only two molecular therapies are currently approved (Trastuzumab for HER2+ GCs and Ramucirumab). To explore in depth the molecular mechanisms sustaining tumor growth and response to therapy, animal models are very useful. At the moment, the best preclinical model to validate targets and positive/negative response predictors of response to therapy is represented by Patient-Derived Xenografts (PDXs), an experimental model that retains the principal histologic and genetic characteristics of the donor tumor, is predictive of clinical outcome and is a valuable tool for personalized medicine decisions. Indeed, this strategy combines the flexibility of preclinical analysis with the informative value of population-based studies. We have recently generated a molecularly annotated colony of gastro-esophageal PDXs (at the moment >90 PDXs) by subcutaneous transplantation in NOD SCID mice. This platform also comprises primary cell lines and 3D-coltured organoids. Although this platform has already been helpful in investigating therapeutic approaches against activated receptor tyrosine kinases, the subcutaneous tumor implantation very rarely allows metastatic dissemination.
To verify if gastric tumors can grow and give rise to metastases when implanted in their original organ, we orthotopically transplanted either cancer cell suspensions or intact cancer tissues. Cancer cell suspensions were inoculated under the serosal coat of mice's stomach while tumor samples were implanted inside the stomach and fixed to the mucosal coat with stitches. Growth of the primary tumor and appearance of metastases have been monitored in vivo using IVIS technology (In Vivo Imaging System, IVIS Spectrum). Pathological analysis has been performed after animal sacrifice.
With both techniques we have been able to observe local tumor growth which was monitored along time through fluorescent 2-Deossi glucose

(Xenolight RediJect 2-DG-750 probe) revealed by IVIS. In animals injected with gastric cells we observed local tumor growth, several intraperitoneal neoplastic lesions and metastatic growth in the lungs. In animals orthotopically transplanted with gastric tumor samples, engraftment was observed and experiments are ongoing to reveal the development of metastases. We intend to use these models to evaluate the efficacy of therapies targeting molecular lesions identified in the implanted tumors. We believe that orthotopic transplantation will better mimic the original microenvironment of the tumor and will thus recapitulate the therapeutic responses observed in patients.
Stomach.

Abstract 109
IMPACT OF PREVIOUS GYNAECOLOGIC MANIPULATION ON SHORT TERM SURGICAL OUTCOME IN PATIENTS TREATED WITH CYTOREDUCTIVE SURGERY (CRS) AND HYPERTHERMIC INTRAPERITONEAL CHEMOTHERAPY (HIPEC)

M. Guaglio, S. Kusamura, D. Baratti, V. Pruiti Ciarello, L. Battaglia, M. Deraco. *Fondazione IRCCS Istituto Nazionale Tumori, Milan, Italy*

CRS/HIPEC is a complex procedure that become even more challenging after previous surgical manipulation, due to adhesions and tumor spread through the scars. In our experience, most of female patients affected by peritoneal surface malignancy (PSM) were previously mistakenly operated, thinking at ovarian cancer. We attempted to assess surgical and prognostic outcome of CRS/HIPEC after previous inappropriate surgeries in this setting.
A prospective database of CRS/HIPEC procedures performed on female patients affected by non gynaecological PSM was reviewed. 222 CRS/HIPEC were analysed concerning risk factors for urologic complications and severe morbidity (NCI-CTCAEv.3). Age, BMI, ECOG performance status, Charlson comorbidity index, previous chemotherapy, prior surgical score (PSS), previous gynaecological operation (PGO), PCI and number of anastomosis were reviewed. Moreover, impact of PGO and PSS on operation time, blood transfusion and in hospital stay was considered.
Among 222 cases, 114 had received PGO. 173 had PSS>0. Sixteen (7,3%) had urologic complications and 78 (35,1%) had severe morbidity G3-5. Factors associated with urologic complications were PGO ($p < 0.001$) and PSS ($p = 0.026$). Independent risk factors for severe morbidity were age >55 years (OR: 2.3; $p = 0.009$) and number of anastomosis (OR: 2.6; $p = 0.002$). PGO was associated with longer length of operation (U test, $p = 0.035$) but not with intraoperative blood transfusion or in hospital stay.
PSS was associated with increased in hospital stay with a borderline significance ($p = 0.052$).
Previous gynaecologic operation in female patients affected by PSM significantly increases urological postoperative complications, such as PSS. For this reason, in carcinosis of unknown origin it becomes mandatory to tailor the diagnostic pathway, avoiding as much as possible uncomplete peritoneal resections, especially in the pelvis.
Peritoneal carcinomatosis.

Abstract 110
DRUG COMBINATIONS FOR HIPEC AFTER CYTOREDUCTIVE SURGERY IN DIFFUSE MALIGNANT PERITONEAL MESOTHELIOMA: A PSOGI REGISTRY STUDY

S. Kusamura [1], D. Barattii [1], P.H. Sugarbaker [2], D. Elias [3], O. Glehen [4], E. Levine [5], D.L. Morris [6], M. De Simone [7], Y. Yonemura [8], M. Deraco [1]. [1]*Fondazione IRCCS Istituto Nazionale Tumori di Milano, Milano, Italy;* [2]*Washington Cancer Institute, Washington Hospital Center, Washington DC, USA;* [3]*Institut Gustave Roussy, Paris, France;* [4]*Centre Hospitalo-Universitaire Lyon Sud, Hospices Civils de Lyon, Lyon, France;* [5]*Wake Forest University, Winston-Salem, USA;* [6]*University of New South Wales Department of Surgery, St George Hospital, Sydney, Australia;* [7]*IRCC Istituto di Candiolo, Torino, Italy;* [8]*NPO to Support Peritoneal Dissemination Treatment, Osaka, Japan*

Aim: To test what is the drug combination for HIPEC linked with the best prognosis in diffuse malignant peritoneal mesothelioma (DMPM) patients after cytoreductive surgery (CRS).

Patients and methods: Five hundred ninety-seven DMPM patients treated with CRS and HIPEC were enrolled to the study. Survival analysis was conducted to test whether the type of HIPEC drug combination is correlated with prognosis. Univariate and multivariate Cox regression models were developed to identify independent predictors of overall (OS) and progression free survivals (PFS).
Results: Four hundred eighty-seven (81.6%) cases had epithelioid histology and the mean peritoneal cancer index (PCI) was 20.1. One hundred seventy-six (29.4%) underwent incomplete cytoreduction, and 75 (12.6%) cases received early postoperative intraperitoneal chemotherapy. The HIPEC drug schedules were distributed as follows: cisplatin alone: 102, cisplatin + doxorubicin: 249, cisplatin + doxorubicin + ifosfamide: 86, cisplatin + mitomycin-C: 54, irinotecan + oxaliplatin: 31, oxaliplatin alone: 37, mitomycin-C: 23, and other agent: 15. Severe postoperative morbidity and mortality rates were 22.2%, and 4.3%, respectively. Median survival was 48.2 months, and 5-year overall survival (OS) rate was 47.1% in the entire series. Risk of death associated with regimens did not differ significantly from each other (Cox univariate). However, platin-based HIPEC resulted to be independently linked with better OS (HR: 0.40, 95% CI: 0.18–0.88) and better PFS (HR: 0.39, CI 95%: 0.19–0.81), according to Cox multivariate analysis. Other independent prognosticators were age (PFS), completeness of cytoreduction (OS), histology (OS/PFS), PCI (OS/PFS), and severe morbidity (OS/PFS).
Conclusion: Platin-based HIPEC combinations seem to be correlated with the best prognostic results in DMPM submitted to CRS.

Abstract 8
PRESSURIZED INTRAPERITONEAL AEROSOL CHEMOTHERAPY (PIPAC) WITH OXALIPLATIN, CISPLATIN AND DOXORUBICIN IN PATIENTS WITH PERITONEAL CARCINOMATOSIS: PRELIMINARY ANALYSIS OF AN OPEN-LABEL, SINGLE-ARM, PHASE II CLINICAL TRIAL

M. Robella, M. Vaira, A. Borsano, M. De Simone. *Unit of Surgical Oncology - Candiolo Cancer Institute, Candiolo, Torino, Italy*

PIPAC is an innovative approach to peritoneal carcinomatosis (PC) that applies chemotherapic drugs into peritoneal cavity as an under pressure air-flow. It improves local bioavailability of cytostatic drugs as compared with conventional intraperitoneal chemotherapy. Aim of this study is to prove feasibility, efficacy and safety of this new approach; hereinafter the first step analysis of this phase II trial.
Patients included for the analysis underwent at least 2 PIPAC procedures; drugs used were Oxaliplatin 92 mg/m2 for colorectal cancers and Cisplatin 7.5 mg/m^2 + Doxorubicin 1.5 mg/m^2 for ovarian, gastric and primary peritoneal cancers. A pressure of 10 mm Hg at 37 °C for 30 min/course. The primary endpoint was the Overall Response Rate according to RECIST criteria after 2 and 3 PIPAC. Secondary significant endpoints were clinical tumor response using FDG-PET according to PERCIST criteria, tumor regression on histology, PC Index improvement on repeated PIPAC and quality of life. Safety and tolerability has been assessed according to CTCAE2. Between June 2015 and March 2017, 133 single-port PIPAC procedures in 63 patients presenting PC from different primary tumors, not eligible for surgery +/- HIPEC, were performed. Thirty-five patients were enrolled. Laparoscopic non-access rate was 1/35 (2.8%). Twenty-five patients (55 PIPAC procedures) were eligible for analysis. Nine patients reported a disease stability, 4 a partial response and 12 patients a progression of disease. Fifteen patients were undergoing systemic chemotherapy (sCT) with a wash-out interval of at least 2 weeks before and 1 week after each PIPAC. Clinical tumor response according con PERCIST Criteria resulted to be a not reliable tool considering the mucinous histology of some patients and the lack of sensitivity in assessing PC. Tumor regression on histology and PC Index improvement were observed in 7/25 (28%) and in 6/25 (24%), respectively. CTCAE grades 1 and 2 were observed after 3 and 5 procedures, respectively, for abdominal pain and nausea. Renal and hepatic functions were not impaired; no cumulative renal toxicity was observed after repeated PIPAC procedures. The association of PIPAC and sCT does not induce significant hepatic and renal toxicity. SF-36 and EORTC QLQ-30 global physical health scores and pain improved during therapy.
Single-port PIPAC resulted to be feasible, safe and easy to perform. This new approach as well as being ethically accepted, may be an useful strategy for patients not eligible to radical surgery, presenting extra-

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Cancer Research

Resource Report

A Comprehensive PDX Gastric Cancer Collection Captures Cancer Cell–Intrinsic Transcriptional MSI Traits

Simona Corso[1,2], Claudio Isella[2], Sara E. Bellomo[2], Maria Apicella[2], Stefania Durando[2], Cristina Migliore[1,2], Stefano Ughetto[1,2], Laura D'Errico[1,2], Silvia Menegon[2], Daniel Moya-Rull[1,2], Marilisa Cargnelutti[2], Tânia Capelôa[2], Daniela Conticelli[1,2], Jessica Giordano[1,2], Tiziana Venesio[2], Antonella Balsamo[2], Caterina Marchiò[2,3], Maurizio Degiuli[4], Rossella Reddavid[4], Uberto Fumagalli[5], Stefano De Pascale[5], Giovanni Sgroi[6], Emanuele Rausa[6], Gian Luca Baiocchi[7], Sarah Molfino[7], Filippo Pietrantonio[8,9], Federica Morano[8], Salvatore Siena[9,10], Andrea Sartore-Bianchi[9,10], Maria Bencivenga[11], Valentina Mengardo[11], Riccardo Rosati[12], Daniele Marrelli[13], Paolo Morgagni[14], Stefano Rausei[15], Giovanni Pallabazzer[16], Michele De Simone[2], Dario Ribero[2], Silvia Marsoni[2], Antonino Sottile[2], Enzo Medico[1,2], Paola Cassoni[3], Anna Sapino[2,3], Eirini Pectasides[17], Aaron R. Thorner[18], Anwesha Nag[18], Samantha D. Drinan[18], Bruce M. Wollison[18], Adam J. Bass[17], and Silvia Giordano[1,2]

Abstract

Gastric cancer is the world's third leading cause of cancer mortality. In spite of significant therapeutic improvements, the clinical outcome for patients with advanced gastric cancer is poor; thus, the identification and validation of novel targets is extremely important from a clinical point of view. We generated a wide, multilevel platform of gastric cancer models, comprising 100 patient-derived xenografts (PDX), primary cell lines, and organoids. Samples were classified according to their histology, microsatellite stability, Epstein–Barr virus status, and molecular profile. This PDX platform is the widest in an academic institution, and it includes all the gastric cancer histologic and molecular types identified by The Cancer Genome Atlas. PDX histopathologic features were consistent with those of patients' primary tumors and were maintained throughout passages in mice. Factors modulating grafting rate were histology, TNM stage, copy number gain of tyrosine kinases/*KRAS* genes, and microsatellite stability status. PDX and PDX-derived cells/organoids demonstrated potential usefulness to study targeted therapy response. Finally, PDX transcriptomic analysis identified a cancer cell–intrinsic microsatellite instability (MSI) signature, which was efficiently exported to gastric cancer, allowing the identification, among microsatellite stable (MSS) patients, of a subset of MSI-like tumors with common molecular aspects and significant better prognosis. In conclusion, we generated a wide gastric cancer PDX platform, whose exploitation will help identify and validate novel "druggable" targets and optimize therapeutic strategies. Moreover, transcriptomic analysis of gastric cancer PDXs allowed the identification of a cancer cell–intrinsic MSI signature, recognizing a subset of MSS patients with MSI transcriptional traits, endowed with better prognosis.

Significance: This study reports a multilevel platform of gastric cancer PDXs and identifies a MSI gastric signature that could contribute to the advancement of precision medicine in gastric cancer.

Introduction

Gastric cancer is the fifth most common cancer and the third leading cause of cancer mortality in the world (1). From a histologic point of view, according to Lauren's classification, it can be divided in three main subtypes: intestinal, characterized by a glandular or papillary structure, frequently originating from intestinal metaplasia; diffuse, showing a poorly cohesive tissue architecture; mixed, presenting areas of both intestinal and diffuse histology. The Cancer Genome Atlas (TCGA) analysis has proposed a molecular classification of this disease (2), recognizing

[1]Department of Oncology, University of Torino, Candiolo, Italy. [2]Candiolo Cancer Institute, FPO-IRCCS, Candiolo, Italy. [3]Department of Medical Sciences, University of Torino, Italy. [4]Department of Oncology, University of Torino, Orbassano, Italy. [5]Chirurgia Generale 2, Spedali Civili, Brescia, Italy. [6]Surgical Oncology Unit, Surgical Science Department, ASST Bergamo Ovest, Treviglio (BG), Italy. [7]Department of Clinical and Experimental Sciences, Surgical Clinic, University of Brescia, Italy. [8]Medical Oncology Department, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy. [9]Department of Oncology and Hemato-oncology, University of Milan, Milan, Italy. [10]Niguarda Cancer Center, Grande Ospedale Metropolitano Niguarda, Milan, Italy. [11]First Department of General Surgery, Borgo Trento Hospital, University of Verona, Verona, Italy. [12]Gastroenterological Surgery Unit, IRCCS San Raffaele Hospital, Vita-Salute University, Milan, Italy. [13]Department of Medicine, Surgery and Neurosciences, Unit of General Surgery and Surgical Oncology, University of Siena, Siena, Italy. [14]Pathology Unit, Morgagni-Pierantoni Hospital, Forlì, Italy. [15]Department of Surgery, University of Insubria, Varese, Italy. [16]Esophageal Surgery Unit, Medical University of Pisa, Pisa, Italy. [17]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts. [18]Center for Cancer Genome Discovery (CCGD) Dana-Farber Cancer Institute, Boston, Massachusetts.

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four different genetic subtypes: Epstein–Barr virus (EBV) positive (9%), microsatellite instable (MSI; 22%), chromosomal instable (50%), and genomically stable (20%). Each subtype is characterized by specific genomic alterations, many of which are potentially targetable.

In spite of the increased molecular knowledge of gastric cancer, only one targeted therapy directed against molecular alterations of tumor cells has been approved so far, namely treatment with trastuzumab in tumors displaying *HER2* amplification (3). Indeed, most phase III clinical trials evaluating molecular drugs in gastric cancer failed, suggesting the need of a more accurate patient selection and of preclinical models to assist clinical development of novel therapeutic strategies (3–10).

The cancer models that better recapitulate the biological characteristics of human tumors are, at present, patient-derived xenografts (PDX). These models are obtained by subcutaneous or orthotopic implantation in immunodeficient mice of small pieces of human tumors, which are propagated to obtain cohorts of animals bearing the same tumors on which preclinical trials (xenotrials) can be performed (11, 12). Xenotrials can be used to (i) validate altered genes as tumor drivers, that is, possible therapeutic targets; (ii) directly compare treatments targeting the same genetic lesions; (iii) identify biomarkers of sensitivity/resistance; and (iv) compare the effect of different cooccurring genetic alterations on the therapeutic response. Indeed, some of these studies performed in PDXs have generated important preclinical information that led to the execution of successful clinical trials (13). Moreover, experience derived from coclinical trials, where treatments have been performed both in patients and in the corresponding PDXs, has shown the power of this approach (14, 15).

Because of the low prevalence of many genetic lesions, however, these studies can be successfully performed only if a large number of PDXs is available. For this reason, we generated a wide gastroesophageal PDX platform (to our knowledge, the widest in an academic institution), encompassing all the histologic and molecular subtypes, also including *in vitro*–derived material, such as primary cell lines and organoids. We believe that this multilevel platform, rapidly and continuously growing, represents an invaluable resource to validate the effectiveness of inhibiting already known drivers, to perform preclinical studies comparing different therapeutic approaches, to identify new targets and to investigate mechanisms of resistance to treatment. The final goal is to generate knowledge to be translated in clinical trials, which can eventually improve the prognosis of this deadly disease.

Materials and Methods

PDX generation

Gastric PDX generation was performed as described in ref. 16. Details are reported in Supplementary Material. All animal procedures adhered to the "Animal Research: Reporting of In Vivo Experiments" (ARRIVE) standards and were approved by the Ethical Commission of the Candiolo Cancer Institute (Candiolo, Torino, Italy), and by the Italian Ministry of Health. 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).

Gastric cancer primary cell lines culture and organoids

Gastric cancer primary cells were derived from PDXs as described in ref. 17. Gastric cancer organoids were derived as described in ref. 18. The genetic identity of the *in vitro*–derived material with the original tumor has been verified by short tandem repeat profiling (Cell ID, Promega). *Mycoplasma* testing was performed upon culture setting.

PDX xenotrials

GTR0503 PDXs were passaged and expanded for 2 generations until production of a cohort of 40 mice. Established and randomized tumors (average volume, 300 mm^3) were treated for the indicated days with the following regimens (either single agent or combination): vehicle (saline) per os; cetuximab 20 mg/Kg, twice weekly i.p.; JNJ-605 50 mg/kg, daily, per os ($n = 6$). 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 minor tumor axis and D is the major tumor axis. GTR0233 and GTR0455 PDXs were expanded for 4 and 2 generations, respectively, until the production of 20 mice for each gastric cancer model. Established tumors were randomized (average volume, 250 mm^3) and treated for the indicated days with vehicle (saline) or trastuzumab 30 mg/kg, weekly, through intraperitoneal injection.

Organoid transplantation

Gastric cancer organoids were subcutaneously injected into flanks of NOD/SCID mice. For each organoid line, three mice were used, each receiving into one flank organoids derived from 24 wells of a 24-well plate ($\sim 2 \times 10^6$ cells/mouse) in 100 μL Matrigel.

Imaging

Hematoxylin and eosin staining was performed on 3-μm thick tissue sections. Organoids were embedded in Richard-Allan Scientific HistoGel Specimen Processing Gel (Thermo Fisher Scientific) according to the manufacturer's instructions, fixed in formalin, processed according to standard methods, and finally embedded in paraffin.

Images were captured with the AxiovisionLe software (Zeiss) using an Axio Zeiss Imager 2 microscope (Zeiss).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Current address for T. Capelôa: FATH - Pole of Pharmacology and Therapeutics; IREC - Institute of Experimental and Clinical Research; UCL - Université Catholique de Louvain, Woluwe-Saint-Lambert, Belgium; current address for U. Fumagalli and S. De Pascale: Chirurgia dell'Apparato Digerente – European Institute of Oncology IRCCS – Milan, Italy; current address for S. Rausei: Department of Surgery, ASST Valle Olona, Gallarate, Varese, Italy; and current address for S. Marsoni: IFOM - The FIRC Institute of Molecular Oncology, Milan, Italy.

Corresponding Authors: Silvia Giordano and Simona Corso, University of Torino, Candiolo Cancer Institute, FPO-IRCCS, Strada Provinciale 142, Candiolo 10060, Italy. Phone: 39011993-3233; Fax: 39011993-3225; E-mail: silvia.giordano@unito.it; and Simona Corso, simona.corso@unito.it

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Cell viability assay

Cells were seeded in 96-well plastic culture plates (3,000/well), in the presence of the indicated drugs or vehicle (DMSO) for 6 days. Cell viability was measured by using the Cell Titer-Glo Luminescent Cell Viability Assay (Promega).

EBV evaluation

Detection and quantification of EBV DNA were performed using the EBV Q-PCR Alert KIT (ELITechGroup S.p.A.). The real-time amplification assay was carried out on ABI 7300 Real-Time PCR System instrument (Applied Biosystems). PDXs were classified as described in ref. 16: EBV high [with high EBV burden, >1,000, 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.

MSI evaluation

Microsatellite stability 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 MSI 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

DNA extracted from PDX models along with a sample of normal germline DNA from each patient were utilized 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 upon their alteration in prior studies of gastroesophageal cancer (19). Details are reported in Supplementary Methods.

Microarray data generation, preprocessing, and differential expression analysis

Synthesis of cDNA and biotinylated cRNA (from 500 ng total RNA) was performed using the IlluminaTotalPrep RNA Amplification Kit (Ambion), according to the manufacturer's protocol. Quality assessment and quantitation of cRNAs were performed with Agilent RNA Kits on a Bioanalyzer 2100 (Agilent). Hybridization of cRNAs (750 ng) was carried out using Illumina Human 48k gene chips (Human HT-12 V4 BeadChip). Array washing was performed by Illumina High Temp Wash Buffer for 10 minutes at 55°C, followed by staining using streptavidin-Cy3 dyes (Amersham Biosciences). Hybridized arrays were stained and scanned in a Beadstation 500 (Illumina) and HiScanSQ. Data were analyzed as described in ref. 16. Details are reported in Supplementary Methods.

Transcript profiling

Array data are deposited in GSE98708 and GSE128459 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=snspycqynfyhful&acc=GSE98708; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE128459).

Statistical analysis

Statistical testing for pharmacologic experiments was performed with GraphPAD PRISM Software 8.0, using the test indicated in the figure legend. Statistical significance: ns = not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Results

Patients' characteristics

Patients' with gastroesophageal cancer were consecutively enrolled in 15 different Italian Hospitals in which the GEA (Gastro-Esophageal Annotated platform) project has been approved. We included in the study a total of 349 patients with gastroesophageal cancer (Fig. 1A), whose tumors were molecularly characterized and whose follow-up was recorded. Detailed patient characteristics are reported in Supplementary Table S1. Median patients' age was 71 (range, 32–90 years), with a male-to-female ratio of 1.97 (226:115). Tumors were located in the gastroesophageal junction (19.1%) or in the upper part of the stomach (6.9%), 19.4% in the middle part, and 49.6% in the lower part; 5% derived from residual tissue of a previous gastrectomy. From a histologic point of view, according to Lauren classification, intestinal, diffuse, and mixed carcinomas were 66.2%, 29.3%, and 4.5%, respectively. Differentiation (defined by grading) was high in 2.6%, moderate in 27.3%, and poor in 70.1% of the cases. In 38.9% of patients, tumors were diagnosed at stages I/II, and in 61.1% at stages III/IV. 21.3% of patients received neoadjuvant chemotherapy before surgery.

Gastroesophageal carcinomas were also analyzed for EBV and microsatellite stability status: 10% of tumors had an intermediate/high EBV burden, whereas 17.6% showed MSI (Supplementary Table S1).

Establishment of PDX models

From the 349 patients included in the study, we established 145 PDX models in NOD/SCID mice, with a success rate of 42% (in the range of what was previously reported; ref. 20). The histologic analysis of PDXs revealed that around 30% of the mice developed a human-derived lymphoproliferative disease (monoclonal and EBV^+), characterized by a mutational burden and an expression profile distinct from gastric adenocarcinomas and endowed with very fast growth kinetics (16). Lymphoma onset did not correlate neither with the level of lymphocyte infiltration, nor with the histotype of the original gastric tumor, nor with patient outcome (16).

The 100 PDXs that developed gastric cancer (Fig. 1A) showed histopathologic features consistent with those of patients' primary tumors that were maintained throughout different passages in mice (Fig. 1B and C). Indeed, hierarchical clustering analysis of the transcriptome confirmed that PDXs were significantly more similar to their corresponding primary tumors than to unmatched pairs (Fig. 1D). These data suggest that PDXs maintained the identity of their preimplantation surgical counterparts, both at histologic and transcriptional levels. Only in few cases, primary tumors with mixed histology generated either intestinal or diffuse PDXs (Fig. 1C), probably as a consequence of an unbalanced representation of the mixed component in the transplanted primary.

The mean latency period of tumor growth (from implant to the appearance of a palpable tumor) was 73.5 days (median, 61 days; range, 27–237 days). In 83% of the PDXs, the latency period shortened in the following serial passages (mean engraftment time at second passage, 50 days; median, 38 days; range, 15–414 days; Supplementary Table S2).

Factors influencing PDX generation

To investigate potential factors influencing PDX generation, we evaluated both patient and tumor characteristics. We did not

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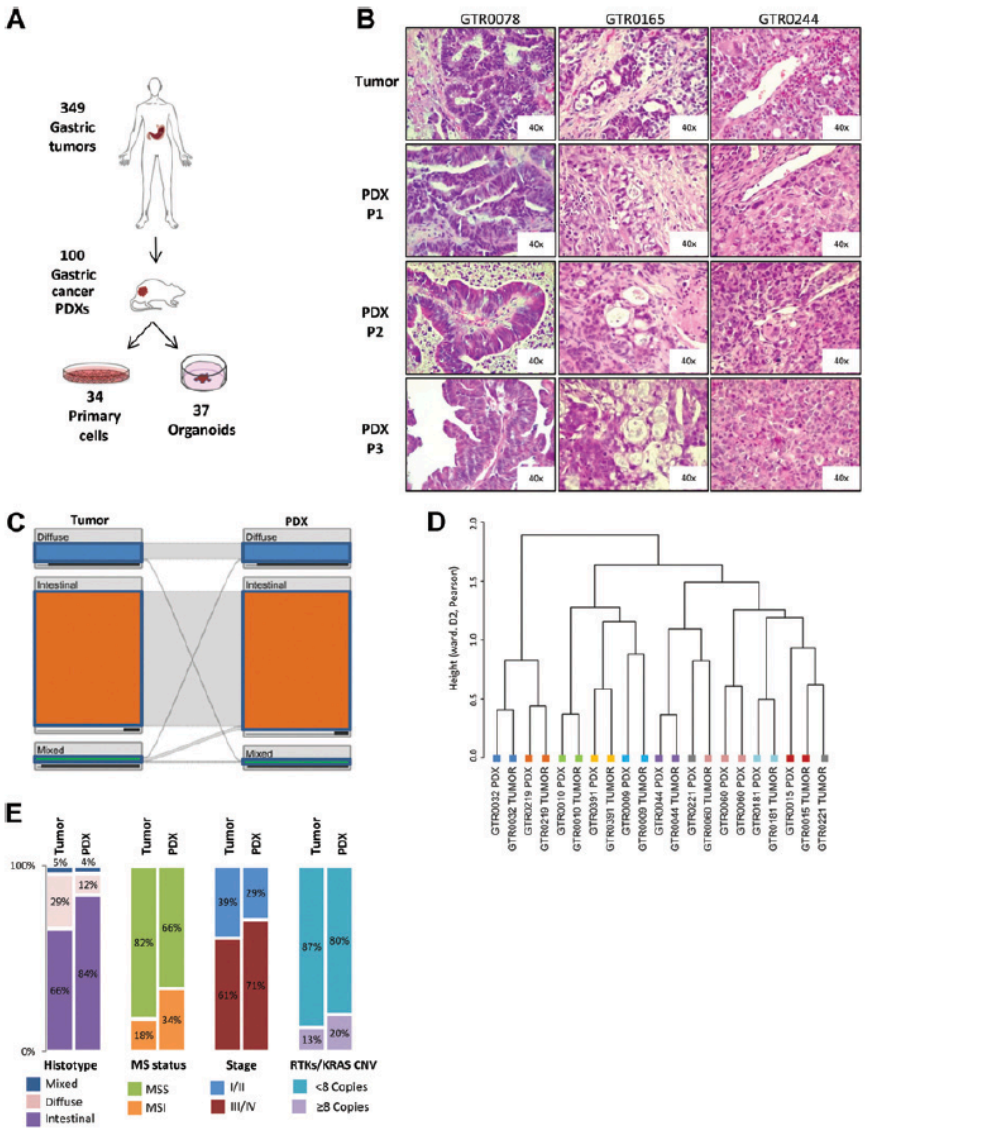

Figure 1.

The PDX platform captures all the gastric cancer subtypes, and it is enriched in intestinal histology, MSI status, high stage, and RTK/KRAS amplification compared with donor tumors. **A,** A total of 349 fresh surgical gastric adenocarcinoma samples were subcutaneously implanted in NOD/SCID mice, generating 100 gastric PDXs; from a fraction of PDXs, we derived 34 2D primary cell lines (from 72 samples) and 37 organoids (from 51 samples). **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 displays also foci of mucin production; GTR0244 shows a diffuse growth pattern. As illustrated, xenografted tumors retained the histopathologic characteristics of the original samples through passages. **C,** Caleydo plot showing the histologic correlation between primary tumor and the corresponding PDX. The graph shows that in the vast majority of the cases, there was a perfect match between the primary tumor and the PDX. **D,** Unsupervised hierarchical clustering analysis of the transcriptome performed on primary tumors and the corresponding PDXs. The analysis demonstrates that PDXs were significantly more similar to their corresponding primary tumors than to unmatched pairs. **E,** The graph illustrates the percentage of donor tumors ($n = 349$) and of derived PDXs ($n = 100$) for the following features: histology (intestinal, diffuse, or mixed); microsatellite stability status (MSI or MSS); stage (I/II or III/IV); RTK/*KRAS* copy number variation (CNV < or ≥8 copies).

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observe any statistically significant correlation between engraftment and patient characteristics such as age or gender. Quite surprisingly, previous neoadjuvant chemotherapy did not significantly affect tumor take rate (Supplementary Table S2). Concerning the pathologic characteristics of the tumors, although tumor site and EBV status did not correlate with engraftment (Supplementary Table S2), histology turned out to be relevant. Not surprisingly, intestinal type tumors showed a significantly higher grafting than diffuse type tumors (intestinal tumors engrafted in 36.52% of the cases vs. 11.34% of diffuse ones, $P < 0.0001$; Supplementary Table S2; Fig. 1E). Other factors influencing PDX generation were tumor–node–metastasis (TNM) stage, copy number gain of genes coding for receptor tyrosine kinases (RTK)/*KRAS*, and MSI status. Concerning TNM, stage III/IV tumors showed a higher engraftment rate ($P < 0.05$; Supplementary Table S2; Fig. 1E). Tumors with a RTK/*KRAS* amplification ($\geq$8 gene copies, a threshold considered biologically and clinically relevant; refs. 21, 22) positively correlated with engrafting. Indeed, 43.47% of primary tumors presenting RTK/*KRAS* amplification engrafted versus 26.75% of the non-amplified ones ($P < 0.05$; Supplementary Table S2). Finally, MSI tumors had an engraftment rate significantly higher than MSS tumors (55.93% vs. 23.64%, $P < 0.0001$); this resulted in the enrichment of MSI PDXs (34%) compared with the donor patient population (18%; Fig. 1E).

PDX characterization

As shown in Fig. 1, the PDX platform captures all the gastric cancer subtypes, even if it is enriched in intestinal histology, MSI status, high stage, and RTK/*KRAS* amplification compared with the donor tumors. As mentioned above, MSI tumors showed an engraftment rate higher than MSS ones. Interestingly, although some "stable" microsatellites were still present in primary tumors, they were completely lost in the corresponding PDXs (Supplementary Fig. S1A and S1B). To investigate whether this was due to the loss of human stroma in the PDXs (which was present in the primary tumors, possibly contributing the "normal" allele) or to the *in vivo* selection of a more unstable subpopulation, we generated organoids from primary tumors and analyzed them after few passages. As already described also by others (23), gastric cancer organoids achieved very high tumor purity, with few or no stroma component (Supplementary Fig. S2A). Indeed, organoid analysis showed the absence of the MSS component (Supplementary Fig. S1B), thus strengthening the hypothesis that the "stable" component is contributed by the human stroma.

As previously mentioned, the TCGA consortium identified four major genomic subtypes of gastric cancer, associated with EBV positivity, MSI status, chromosomal instability, and genomic stability, respectively. The integration of data obtained by genomic sequencing, EBV testing, and microsatellite stability evaluation allowed PDX molecular categorization. As shown in Fig. 2A, all the subtypes were captured in the platform, even if the PDX collection displayed a higher occurrence of MSI samples. The analysis of the most frequent genetic alterations (mutations and CNV; Fig. 2B and C) revealed that the PDX platform captures the molecular heterogeneity of human gastric tumors. Indeed, all the most frequent mutations/CNVs reported by TCGA (2) are present in the platform. As the platform comprises several models bearing alterations in druggable genes of the RTK/RAS and RTK/PI(3)K signaling pathways (Fig. 2D; Supplementary Table S3), it is an optimal instrument to perform "xenotrials" verifying the effect of the inhibition of these targets and optimizing the therapeutic approach. PDX models data and metadata will be openly available in PDX Finder (https://doi.org/10.1093/nar/gky984, pdxfinder.org) and in the EurOPDX data portal (http://dataportal.europdx.eu) that will be constantly updated with the newly generated models.

"Xenotrials" with gastric cancer PDXs mimic patient response to targeted therapies

To verify whether our gastric cancer PDXs reliably recapitulate patients' response to targeted drugs, we looked for established PDX in which the corresponding donor patients had been treated with trastuzumab (at present, the only molecular therapy targeting tumor cells approved in gastric cancer). Only one patient bearing $HER2^+$ gastric cancer, from which we derived a PDX, underwent trastuzumab treatment (in combination with chemotherapy), showing primary resistance to this therapy (progressive disease, according to RECIST 1.1 criteria; Fig. 3A; top). The corresponding PDX, named GTR0455, was serially passaged in mice until six tumor-bearing animals were produced per experimental group. When xenografts reached an average volume of approximately 250 mm^3, mice were randomized into 2 cohorts, and treated for one month with either vehicle (saline) or trastuzumab. To assess tumor response to therapy, we measured tumor volume and used a "RECIST 1.1-like" classification, inspired by clinical criteria, already described for PDX models (24): (i) partial response (PR) was defined as a decrease of at least 50% in the tumor volume, taking as reference the baseline volume; (ii) progressive disease (PD) was defined as at least 35% increase in tumor volume; (iii) intermediate tumor variations were defined as stable disease (SD); and (iv) complete response (CR) was the disappearance of the tumor. In accordance with the clinical history of the donor patient (Fig. 3A), all the trastuzumab-treated GTR0455 mice were resistant to treatment and experienced disease progression (Fig. 3A, bottom). Importantly, we observed response to trastuzumab in other $HER2^+$ PDXs (e.g., in GTR0233, reported in Fig. 3B), but we could not compare it to the donor patients, who were never treated with trastuzumab because they never relapsed.

Generation and characterization of PDX-derived cell lines and organoids

To perform *in vitro* studies, from a fraction of the PDXs, we derived both primary cell lines and organoids, obtaining 34 (from 72 samples) 2D primary cell lines and 37 (from 51 samples) organoids (Fig. 1A; for details see Materials and Methods). Histologic analysis of the organoids confirmed that they maintained the characteristics of the corresponding primary tumors and of the PDXs (representative examples are shown in Fig. 4A). Interestingly, when organoids were reinjected in mice, they originated tumors very similar to the corresponding PDXs (Supplementary Fig. S2B). To further characterize the *in vitro* derivatives, we profiled their gene expression. As shown in Fig. 4B, hierarchical clustering analysis of the transcriptome confirmed that both primary cells and organoids very closely recapitulated the PDX of origin.

To confirm the experimental value of these models for testing the responsiveness to targeted drugs matching actionable genomic alterations, we performed *in vitro* and *in vivo* experiments. As an experimental and representative model, we chose a case (GTR0503) displaying amplification (30 copies; Fig. 4C) and overexpression (Fig. 4D) of the *MET* oncogene, encoding the

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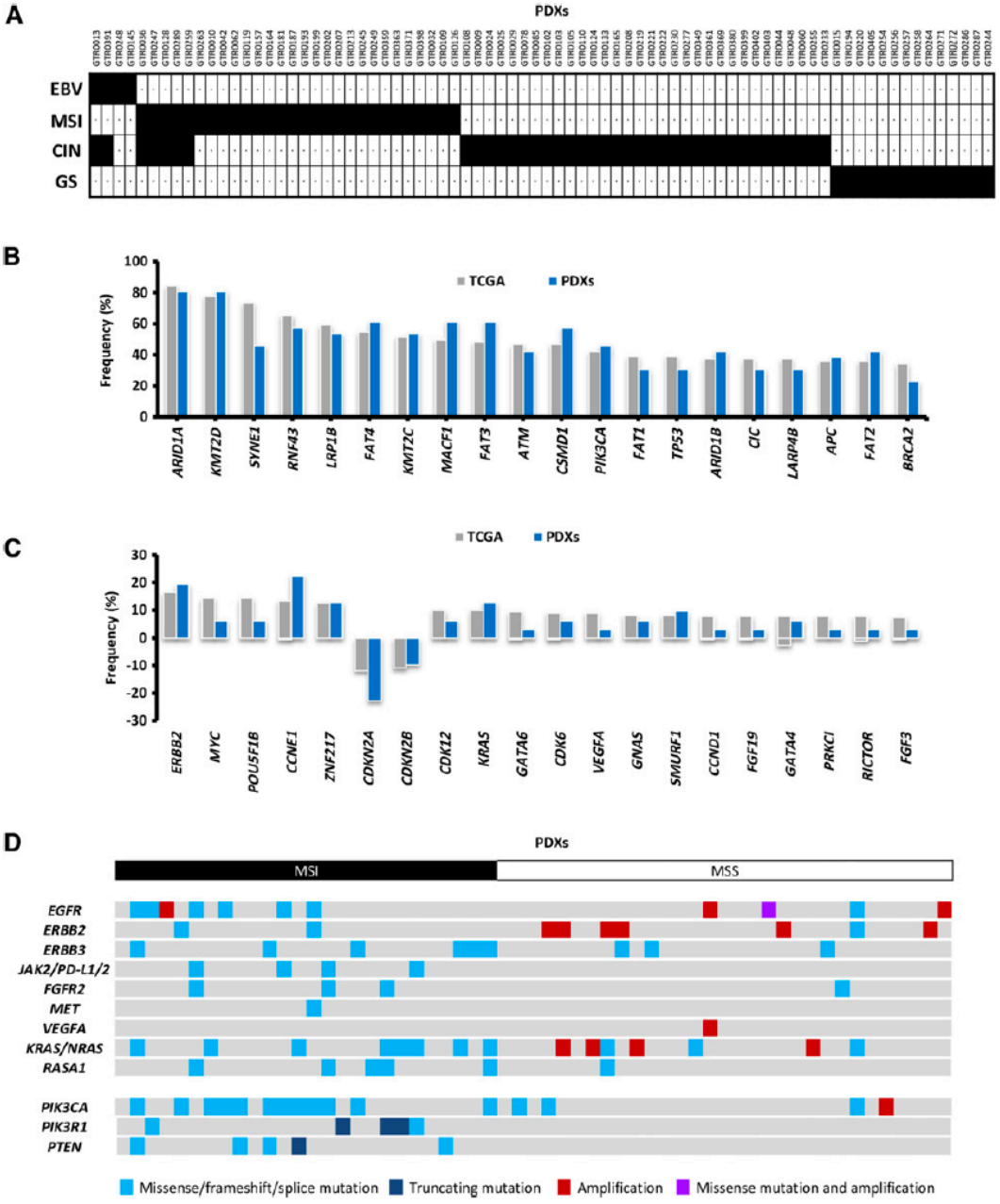

Figure 2.

The PDX platform captures the molecular complexity of gastric cancer. **A,** PDXs were categorized into the molecular subtypes identified by TCGA: EBV-positive, MSI, chromosomal instability (CIN), and genomically stable (GS). **B,** The 20 genes most frequently mutated in MSI tumors in the TCGA dataset (gray bars) and in MSI PDXs (blue bars). **C,** The 20 genes most frequently amplified/lost in non-MSI tumors according to TCGA (gray bars) and their alteration frequency in non-MSI PDXs (blue bars). Amplifications are shown as positive frequencies; deletions as negative values. **D,** Mutations and copy number changes for select genes belonging to RTK/RAS and RTK/PI(3)K signaling pathways are shown across MSI and MSS PDXs.

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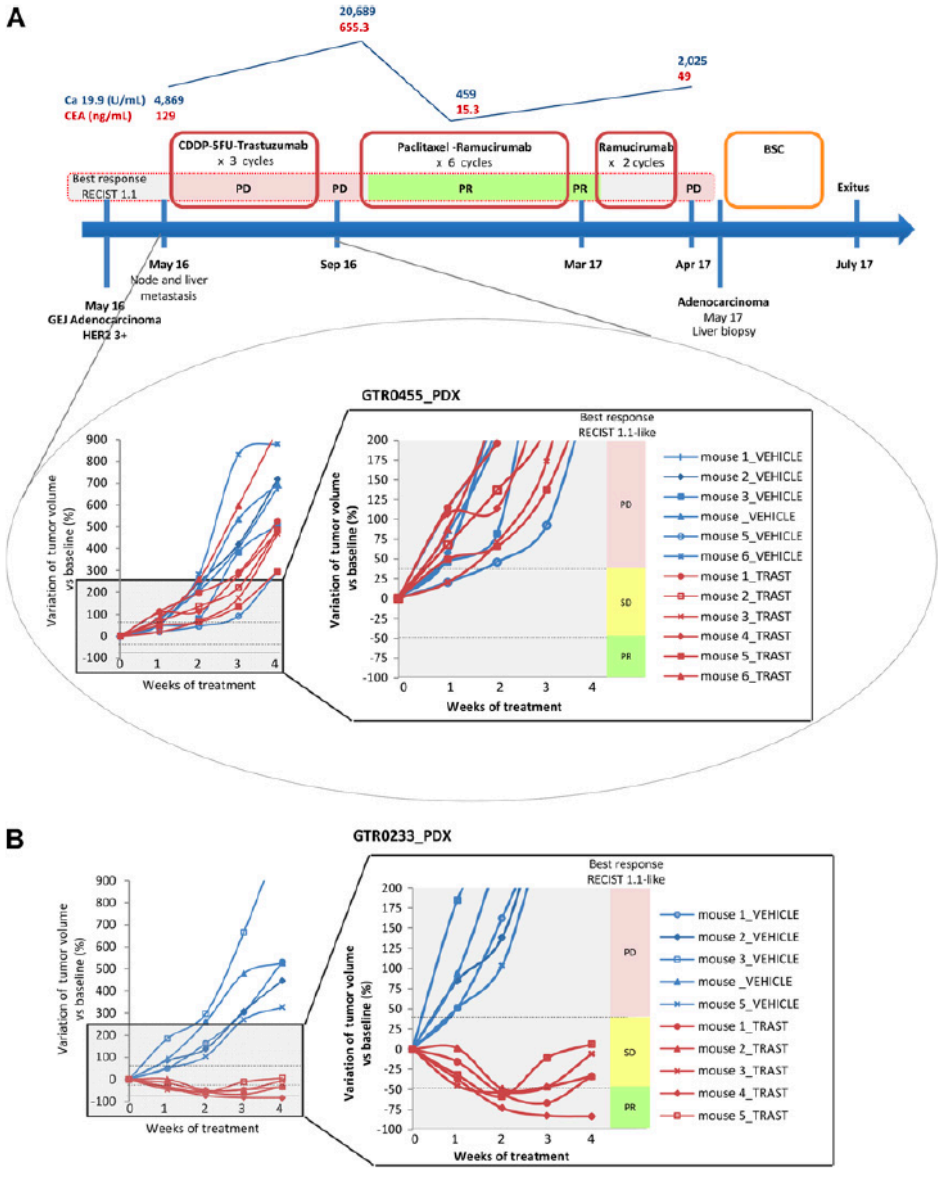

Figure 3.

"Xenotrials" with GC PDXs mimic patient's response to HER2-targeted therapy. **A,** Top, summary of donor patient's clinical history. The clinical course of the patient with HER2$^+$ gastric cancer is summarized, with level of serum cancer antigen 19-9 (Ca 19.9) tumor marker shown throughout treatment (blue line). CEA tumor marker values are reported in red. Red-lined boxes indicate periods of administration of the indicated therapeutic agents. Blue vertical lines indicate timing of tumor specimen acquisition from surgical procedures or biopsies, as well as dates of tumor assessment by either CT scan or FDG-PET/CT scan. PD, progressive disease; PR, partial response, according to RECIST 1.1. BSC, best supportive care. The patient showed primary resistance to trastuzumab treatment. Bottom, Spaghetti plot illustrating the xenotrial performed on the cohort of mice derived from PDX GTR0455, obtained from the above-described donor patient. Individual lines represent, for each mouse, the percentage variation in tumor burden, from treatment start (day 0) to 4 weekly consecutive serial assessments. Blue lines, vehicle-treated mice; red lines, trastuzumab-treated mice (30 mg/kg). The response in mice was evaluated using RECIST 1.1–like criteria, highlighted in the magnification: PD, ≥35% increase from baseline; PR, ≥50% reduction from baseline; stable disease (SD), intermediate variations from baseline. As shown, all mice displayed progressive disease. **B,** Spaghetti plot (performed as in **A**) illustrating the xenotrial performed on the cohort of mice derived from the HER2+ PDX GTR0233. As shown, all mice displayed response to treatment.

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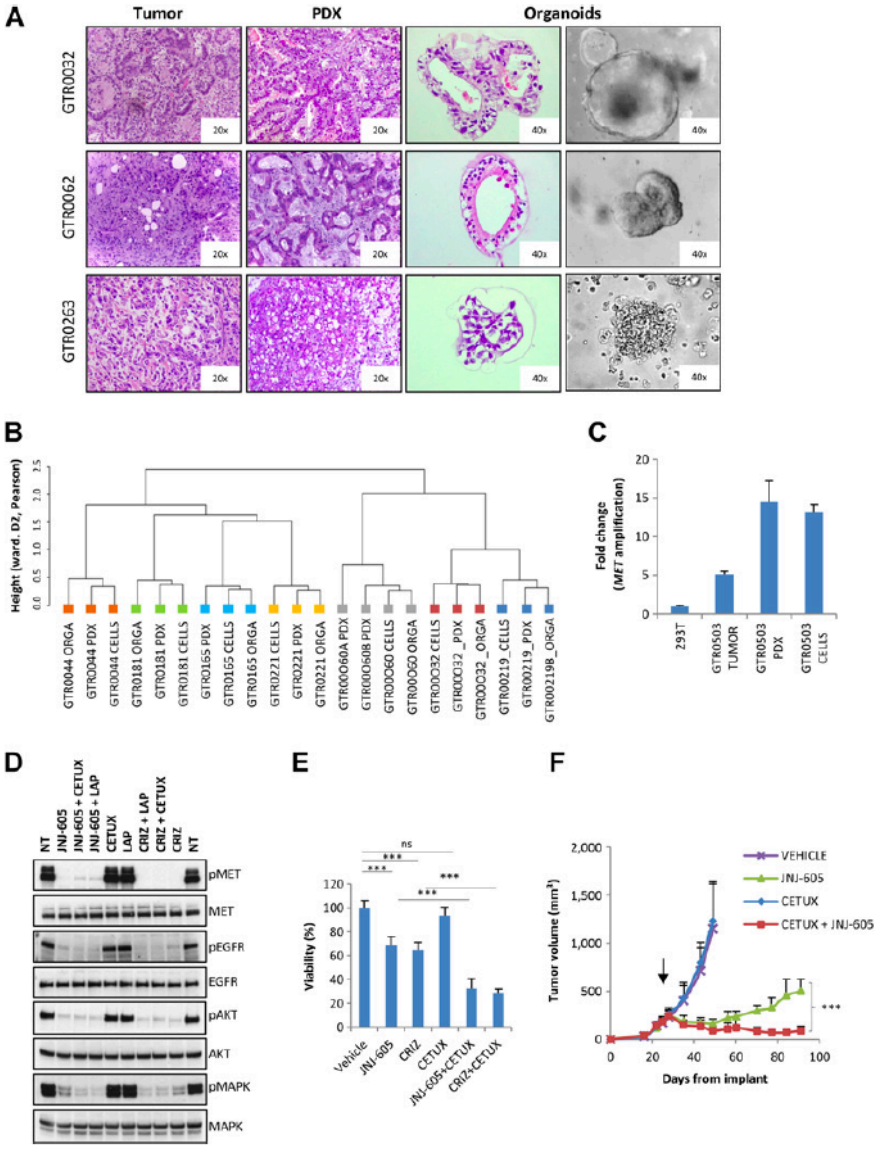

Figure 4.

Generation and characterization of PDX-derived primary cell lines and organoids. **A,** Representative hematoxylin and eosin images and brightfield microscopy of PDX-derived gastric tumor organoids, matched PDXs, and primary tumors for intestinal (GTR0032 and GTR0062) and diffuse histotypes (GTR0263). **B,** Unsupervised hierarchical clustering analysis of the transcriptome performed on organoids (ORGA), primary cells (CELLS), and the corresponding PDXs. The analysis shows the perfect matching of PDXs and the corresponding *in vitro* derivatives. **C,** qRT-PCR analysis of *MET* gene copy number in the original tumor (GTR0503), in the PDX, and in the *in vitro*–derived primary cells compared with the diploid cell line 293T. **D,** Western blot analysis of MET, EGFR, AKT, and MAPK expression and phosphorylation in GTR0503 cells untreated or treated with the indicated drugs (JNJ-605, 250 nmol/L; crizotinib, 250 nmol/L; lapatinib, 250 nmol/L for 2 hours; cetuximab, 10 μg/mL for 16 hours. **E,** Cell viability assay performed on tumor-derived cells, upon treatment with the indicated drugs for 6 days. JNJ-605 (50 nmol/L); CRIZ, crizotinib (50 nmol/L); CETUX, cetuximab (1 μg/mL). **F,** Tumor growth curves in the mice cohort derived from the GTR0503 patient treated with placebo (vehicle), the MET inhibitor JNJ-605 (50 mg/kg, daily, orally), and cetuximab (CETUX, 20 mg/kg, twice weekly i.p.), alone or in combination, as indicated. $n = 6$ mice for vehicle, JNJ-605, and cetuximab arms; $n = 5$ for the combination arm. Arrow, treatment start. **C–F,** Data are represented as mean + SD. ***, $P < 0{,}001$; ns, not significant. One-way ANOVA with Bonferroni multiple comparisons test was used for **E**. Two-way ANOVA followed by Bonferroni multiple comparisons test was used for **F**.

receptor for the HGF. Treatment of primary cells with MET tyrosine kinase inhibitors (JNJ-605, a MET-specific kinase inhibitor; crizotinib, a multikinase inhibitor) resulted in partial inhibition of cell viability. As we showed that in gastric cancer EGFR activation can mediate resistance to MET inhibitors (17), we cotreated the cells with MET and EGFR inhibitors. The dual MET/EGFR targeting resulted both in a sustained inhibition of downstream targets and in a profound impairment of cell growth (Fig. 4D and E). To validate *in vivo* these results, the original tumor was serially passaged to originate 4 independent treatment cohorts (6 PDXs/group): (i) vehicle (placebo); (ii) JNJ-605 (a selective MET inhibitor); (iii) cetuximab; (iv) JNJ-605 + cetuximab. As shown in Fig. 4F, the GTR0503 PDX showed a partial response upon MET inhibition but the addition of the anti-EGFR drug (per se ineffective) resulted in a more intense and prolonged response. These results confirm the experimental predictive value of the *in vitro*–derived models but they also show that the preclinical experiments performed in PDXs can be more informative, providing information also on the long-term response to the treatment.

Identification of a cancer cell–intrinsic MSI signature, which predicts disease outcome

Even if, overall, gastric cancer is endowed with poor prognosis, prognostic heterogeneity has been observed in patients bearing tumors of different molecular subtypes. Indeed, Cristescu and colleagues have shown that patients with MSI tumors display the best prognosis, in line with what observed in other cancer types (25). It is in fact believed that the high mutational burden present in MSI tumors promotes leucocyte infiltration, leading to activation of the immune system (26).

Although the molecular landscape of MSI versus MSS tumors has already been investigated in other tumor types (27), not much is known in the case of gastric cancer. We thus took advantage of the gastric cancer PDX platform to identify genes modulated in MSI cancer cells. In fact, as the stromal component of PDXs is of murine origin, this analysis allows the identification of the molecular differences restricted to the human tumor cells. Focusing on genes expressed by cancer cells, we identified a MSI signature composed of 123 genes with strong differential regulation ($P_{adj} < 0.05$, $|l2r|>1$), subdivided in two modules: 23 of them were upregulated and 100 downregulated (Supplementary Table S4, top; Fig. 5A). As expected, *MLH1* was among the genes strongly downregulated in MSI samples. Interestingly, GSEA analysis showed that most of the dysregulated genes are involved in metabolism and that MSI tumors displayed an increased Warburg phenotype (Supplementary Table S4; bottom).

To predict the MSI status, we calculated a gastric MSI score as the weighted average of the expression of the two modules (Fig. 5B, left; ROC AUC = 0.971). To validate the identified gastric MSI score, we interrogated the largest available gastric cancer gene expression dataset (2) and found that this signature predicted microsatellite instability with high specificity (ROC AUC = 0.93; Fig. 5B, middle). Interestingly, our MSI signature outperformed the previously published MSI signature (27) derived from the analysis of primary human colon cancers that displayed lower prediction values both in the PDX collection and in the TCGA cohort (PDX AUC = 0.86, TCGA ROC AUC = 0.89; Supplementary Fig. S3).

Our gastric MSI score was successfully validated also in the ACRG dataset (AUC = 0.804; Fig. 5B, right; ref. 25). As this dataset is annotated with disease-free survival of patients with gastric cancer, we verified whether our MSI score was associated with a prognostic value. As shown in Fig. 5C, left, the MSI score was indeed able to identify patients with lower recurrence rate (log-rank χ^2 $P < 0.005$). Similar results were obtained also in the GSE26253 (432 patients) and in our PDX cohort as well (IRCC, 65 patients; Fig. 5C, middle and right, respectively). In the ACRG dataset (for which the microsatellite stability status is available), we observed that a portion of MSS samples was endowed with MSI transcriptional traits; strikingly, these patients displayed better prognosis, compared with the other MSS patients (log-rank χ^2 $P < 0.05$; Fig. 5D, left). Moreover, also in the MSI subtype, it was possible to discriminate between patients harboring high or low levels of MSI-like score; despite not reaching significant values (possibly due to the low number of samples), the two populations showed different overall survival (Fig. 5D, right).

Altogether these results demonstrate that the transcriptomic analysis of gastric cancer PDXs allowed the identification of a cancer cell–intrinsic MSI signature, generated without taking in consideration the contribution of leucocyte infiltration. This signature can be efficiently exported to gastric cancer, allowing the identification, among MSS patients, of a subset of MSI-like tumors with common molecular assets and significant better prognosis.

Discussion

Oncology has recently and rapidly moved from a phenotype-based empirical management to a more personalized approach centered on treatment of patients according to their tumor genetic profile. This approach has led to significant results in neoplasms such as lung, breast, and colorectal carcinomas, where driver genes to which tumor cells are addicted have been identified. Unfortunately, this approach has been quite limited in gastric cancer where only two drugs, trastuzumab and ramucirumab, respectively targeting HER2 and VEGFR, have been approved so far (3). For this reason, there is an urgent need for studies able to identify targetable drivers in this neoplasm.

Patient-derived xenografts have proved to be a crucial experimental model to discover new targets in several solid tumors and to be endowed with a high predictive value (11, 12). Although PDXs possess notable advantages, they do have limitations such as their low engraftment rate and poor propensity to metastasize, the presence of a microenvironment that is different from that of the primary tumor, the existence of intratumor heterogeneity, and the engraftment in mice that have a severely compromised immune system. Nevertheless, PDX models represent a significant challenge for oncology research as they reflect human tumor biology more accurately than any other existing models.

We thus generated a platform of gastric cancer PDXs to identify and validate targets and optimize molecular treatments in this disease. To our knowledge, our gastric PDX platform is the widest developed in an academic institution. As most of the molecular alterations that can be investigated as possible therapeutic targets are present only in a minority of gastric cancer samples, the availability of a high number of PDX models is critical for the success of these studies. From the TCGA analysis, in fact, we can infer that the frequency of molecular alterations of targetable kinases such as EGFR, FGFR2, and MET is around or lower than 10% (2). Because in many described samples, either the identified

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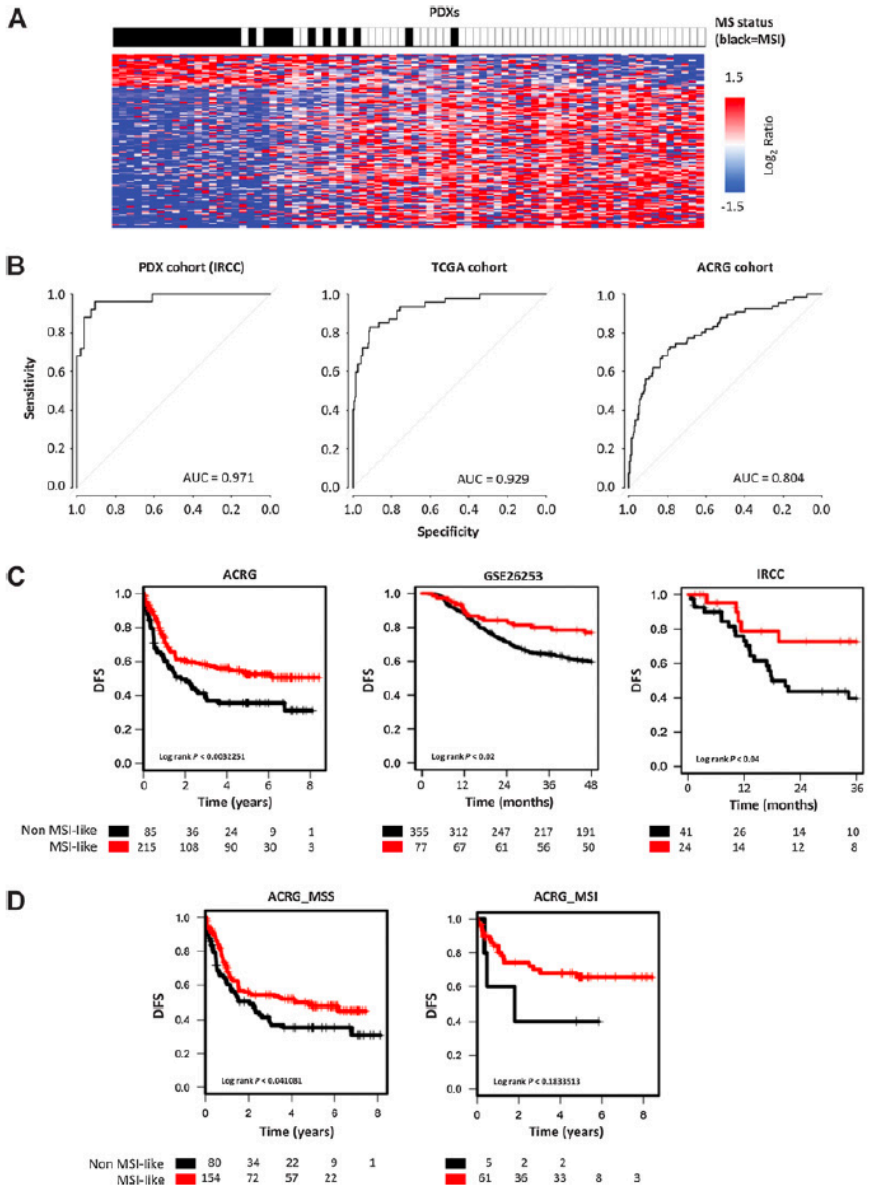

Figure 5.

PDX-derived MSI score predicts outcome in patients with gastric cancer. **A,** Heatmap representing $\log_2$ ratio value of the MSI signature in the PDX collection; top, genetic microsatellite stability (MS) status of the respective tumors is reported (MSI tumors, black rectangles; MSS tumors, white rectangles). **B,** ROC curves of gastric MSI score predicting MSI genetic status in PDX (left), TCGA (middle), and ACRG (GSE66229) cohorts (right). **C,** Kaplan–Meier plot of disease-free survival (DFS) for MSI signature subtyping in prognostically annotated gastric cancer gene expression datasets: ACRG (GSE66229; left), GSE26253 (center), and IRCC (right). **D,** Kaplan–Meier plot of disease-free survival for MSI signature subtyping in the ACRG MSS (left) and MSI (right) subsets.

mutations are not activating or the gene of interest is amplified at a level not sufficient to induce addiction of cancer cells, the number of PDXs suitable to perform preclinical trials is even lower. The availability of a wide PDX platform is thus critical to perform studies on a significant number of models sharing the same genetic lesions.

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The preclinical value of such a platform relies on some requirements: (i) it has to include the whole spectrum of the described histotypes; (ii) the PDXs must recapitulate the histopathologic, biologic, and genetic features of their donor tumors. In this work, we show that our platform includes intestinal, diffuse, and mixed subtypes, as described by Lauren's classification. Even though other groups did not obtain PDXs from tumors of the diffuse subtype (20), we succeeded in establishing PDXs also from this histotype, that maintained their pathologic characteristics. Their under representation is probably due to the fact that these tumors are characterized by the presence of an abundant stroma, containing relatively few cancer cells that are not sufficient to confer a high engraftment rate. Overall, the generated PDX models retained the principal characteristics of donor tumors, including fine tissue structure and subtle microscopic details (gland architecture, mucin production, etc.). Gene expression profile was well conserved among the original tumors, PDXs, and the *in vitro*–derived material. Moreover, the integration of genetic analysis, EBV evaluation, and microsatellite status showed that all the molecular types identified by the TCGA classification were indeed represented. Finally, the genetic analysis revealed that all the most frequent gastric cancer–based genomic alterations identified in public consortia were well represented in our platform. Altogether, these results demonstrate that the platform captures the heterogeneity of human gastric tumors.

The overall engraftment rate of our PDXs (42%) was in line with that described by other authors (20). However, we observed that some characteristics of the tumor could sensibly affect it. Indeed, the histology (intestinal vs. diffuse), the stage (advanced vs. early), the presence of alterations in RTK/*KRAS* genes and MSS status (MSI vs. MSS) significantly increased tumor engraftment, which has been correlated with tumor aggressiveness.

An important feature of PDX models is their ability to mimic patients' response to targeted therapies (12). For gastric cancer PDXs, this is particularly difficult to be verified as, at present, the only approved molecular therapy targeting tumor cells is trastuzumab, an anti-HER2 mAb administered to $HER2^+$, metastatic gastric cancer patients, which represent a relative small fraction (10%–20%) of patients with gastric cancer (3). Only one of our $HER2^+$ PDXs has been derived from a patient who underwent trastuzumab treatment and showed primary resistance; notably, the established PDX completely recapitulated the absence of response observed in the corresponding patient. Importantly, we observed response to trastuzumab treatment in other $HER2^+$ PDXs, but unfortunately, we could not compare it to the donor patients, as they have not been treated with the drug because they never relapsed. However, these results confirm also in PDXs the association between *HER2* amplification and sensitivity to trastuzumab, known to occur in patients.

As a complement to the PDX platform, we also derived *in vitro* primary cell lines and organoids, to allow the execution of biochemical and pharmacologic studies. Also in this case, histologic and gene expression analyses were concordant with those performed in the corresponding PDXs. As already described by others, gastric cancer organoids grown in Matrigel achieve a very high tumor purity, containing few or no stroma (23). As recently highlighted (28), this allows a clear delineation of the cancer cell molecular and transcriptional features, otherwise confounded by normal cells contamination. At the same time, the loss of the stroma component represents a limit. Novel techniques to obtain cancer organoids containing fibroblasts and immune components have been recently proposed (29, 30); in the future, it will be extremely interesting to add these novel models in our organoid collection. Despite the absence of stromal component, 3D cultures are anyway different from classical 2D cultures, as organoids better mimics the physical features and the architecture of the original (solid) tumors; indeed, cancer cells maintain the original morphology and polarity. On the other hand, 2D are less expensive and are an easier experimental system (specially to perform high-throughput assays). For this reason, we decided to derive both 2D and 3D primary cultures from gastric PDXs. Interestingly, for some models, we could obtain both 2D and 3D derivatives, whereas in other cases, we generated only one of the two.

As already demonstrated in other systems, we showed that the pharmacologic response obtained in primary cells and in organoids paralleled what observed *in vivo* (23, 31, 32). However, at least for what concerns molecular therapies targeting RTKs, studies performed in animals can be more informative as they allow the evaluation of the efficacy of long-lasting treatments, show the effectiveness of treatment in delaying/preventing relapse, and are of invaluable value in discovering molecular mechanisms sustaining resistance. Another important point is that targeted drugs often show a different activity *in vitro* and *in vivo*, particularly in the case of some mAbs. Moreover, *in vitro* models are devoid of tumor stroma, which can mediate resistance to tyrosine kinase inhibitors (33).

Finally, we interrogated our platform to gain more insight in MSI tumors. The first observation is that even if MSI gastric cancers display a better outcome, they are intrinsically more aggressive, as testified by their engraftment rate, more than 2-fold higher than that of MSS tumors. The oximorum between these two contradictory observations is probably due to the fact that MSI tumors are characterized by a high mutational burden, which in humans promotes the activation of the immune system; this, in turn, likely mitigates the aggressiveness of the tumor. As our PDXs have been generated in nonimmunocompetent animals, the inhibitory activity of the immune system is lost and, thus, MSI tumors can probably unleash their full aggressiveness.

Both computational methods that analyze next-generation sequencing data and transcriptomic analysis have been developed to detect MSI (27, 34, 35). We decided to exploit the transcriptome of our PDXs to generate a signature able to discriminate MSI and MSS gastric tumors. The use of PDX-derived material has the enormous advantage of taking in consideration only cancer cell–derived material as the stroma is of murine origin and can thus be easily subtracted during the analysis. The possibility to ignore the stromal contribution is very important in gastric cancer where the two main subtypes, intestinal and diffuse, strongly differ for the relative amount of the stromal component, which is significantly more abundant in the latter. In particular, MSI tumors are usually very rich in immune cells whose gene expression importantly affects the transcriptome. The transcriptomic analysis performed on our PDX cohort allowed the identification of a cancer cell–intrinsic MSI signature. In line with our data that show a cell-intrinsic difference between MSI and MSS cancer cells, recent articles have demonstrated that WRN silencing is lethal in MSI cells but not in MSS ones, in the absence of any influence of the microenvironment (36–39). A pillar of current precision oncology is to deconvolve cancer cell–intrinsic oncogenic dependencies and drug resistance mechanisms from microenvironment-driven ones. Exploration of cancer cell transcriptome in PDX takes

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advantage of species-specific sequences to achieve such deconvolution.

Our signature was validated in our and in two wide external datasets. In the ACRG dataset, we verified that our MSI score was able to identify patients with lower recurrence rate; notably, it also identified some patients bearing MSS tumors endowed with MSI transcriptional traits who displayed better prognosis. This observation is important from a clinical point of view as it would allow the identification of cases lacking the genetic MSI characteristics but displaying an MSI-like signature, thus broadening the therapeutic base for Immuno or other PARP-type drugs.

In summary, we have generated a wide gastric cancer PDX platform, which covers in a reliable manner all the gastric cancer subtypes. Its deep molecular annotation as well as the generation of *in vitro*–derived material (primary cells and organoids) represents an invaluable instrument to identify new molecular targets and to optimize therapeutic approaches in gastric cancer.

Disclosure of Potential Conflicts of Interest

C. Marchiò is an advisor at Roche, Bayer, Daichii Sankyo, and MSD. F. Pietrantonio reports receiving other commercial research support from BMS and has received speakers bureau honoraria from Roche, Bayer, Servier, Amgen, Sanofi, Merck Serono, and Lilly. F. Morano has received speakers bureau honoraria from Servier. A. Sartore-Bianchi has received speakers bureau honoraria from Amgen, Bayer, and Sanofi. A. Sapino has received speakers bureau honoraria from Roche. A.R. Thorner is a freelance consultant at AlphaSights. A.J. Bass is a consultant at Lilly, reports receiving commercial research grant from Novartis, Bayer, and Merck, and has ownership interest (including patents) in Signet Therapeutics. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: S. Corso, E. Medico, S. Giordano
Development of methodology: S. Corso, C. Isella, M. Apicella, C. Migliore, D. Conticelli, T. Venesio, E. Medico, S. Giordano
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Ughetto, S. Menegon, C. Marchiò, M. Degiuli, R. Reddavid, U. Fumagalli, S. De Pascale, G. Sgroi, E. Rausa, G.L. Baiocchi, S. Molfino, F. Pietrantonio, F. Morano, S. Siena, A. Sartore-Bianchi, M. Bencivenga, V. Mengardo, R. Rosati, D. Marrelli, P. Morgagni, S. Rausei, G. Pallabazzer, M. De Simone, D. Ribero, A. Sottile, A.R. Thorner, A. Nag, S.D. Drinan, A.J. Bass
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Isella, S.E. Bellomo, J. Giordano, T. Venesio, C. Marchiò, E. Medico, E. Pectasides, A.R. Thorner, A. Nag, B.M. Wollison, A.J. Bass
Writing, review, and/or revision of the manuscript: S. Corso, C. Isella, M. Apicella, C. Marchiò, M. Degiuli, U. Fumagalli, F. Pietrantonio, S. Siena, M. Bencivenga, R. Rosati, D. Ribero, S. Marsoni, E. Medico, E. Pectasides, A.R. Thorner, S. Giordano
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Capelôa, A. Balsamo, P. Morgagni, A. Sottile
Study supervision: S. Corso, S. Giordano
Other (*in vivo* experiments): M. Apicella, S. Durando
Other (performed *in vitro* experiments): C. Migliore, D. Conticelli
Other (molecular and *in vitro* experiments): L. D'Errico
Other (*in vivo* experiments and immunofluorescence assay): D. Moya-Rull
Other (*in vivo* experiments and molecular analysis): M. Cargnelutti
Other (evaluation of the study protocol as coordinator of GIRCG Scientific Committee): D. Marrelli
Other (EBV analyses): A. Sottile
Other (performed the pathological analyses): P. Cassoni, A. Sapino

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REVIEW ARTICLE

Patient-Derived Orthotopic Xenograft models in gastric cancer: a systematic review

Rossella Reddavid[1] · Simona Corso[2,3] · Daniel Moya-Rull[3] · Silvia Giordano[2,3] · Maurizio Degiuli[1]

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Abstract
Patient-Derived Xenografts (PDXs) are, so far, the best preclinical model to validate targets and predictors of response to therapy. While subcutaneous implantation very rarely allows metastatic dissemination, orthotopic implantation (Patient-Derived Orthotopic Xenograft—PDOX) increases metastatic capability. Using a modified tool to analyze model validity, we performed a systematic review of Embase, PubMed, and Web of Science up to December 2018 to identify all original publications describing gastric cancer (GC) PDOXs. We identified ten studies of PDOX model validation from January 1981 to December 2018 that fulfilled the inclusion and exclusion criteria. Most models (70%) were derived from human GC cell lines rather than tissue fragments. In 90% of studies, the implantation was performed in the subserosal layer. Tumour engraftment rate ranged from 0 to 100%, despite the technique. Metastases were observed in 40% of PDOX models implanted into the subserosal layer, employing either cell suspension or cell line-derived tumour fragments. According to our modified model validity tool, half of the studies were defined as unclear because one or more validation criteria were not reported. Available GC PDOX models are not adequate according to our model validity tool. There is no demonstration that the submucosal site is more effective than the subserosal layer, and that tissue fragments are better than cell suspensions for successful engraftment and metastatic spread. Further studies should strictly employ model validity tools and large samples with orthotopic implant sites mirroring as much as possible the donor tumour characteristics.

Keywords Gastric cancer · Stomach tumour · PDOX · Orthotopic transplantation · PDX · Model validity tool

Introduction

Gastric cancer (GC) is the third leading cause of cancer mortality worldwide (8.2%) after colorectal (9.2%) and lung (18.4%) cancers in both sexes combined [1]. In spite of relevant improvements in surgery and preoperative and adjuvant chemotherapy, advanced and metastatic GC still show poor prognosis. Recent advances in knowledge of GC molecular biology and related signaling pathways provide hopeful strategies for targeted therapies treatment of the disease. So far, only two molecular therapies are worldwide approved: Trastuzumab (for HER2 + GCs) and Ramucirumab (targeting angiogenesis) [2, 3]. Very recently, Pembrolizumab has been approved as an immunostimulatory treatment in PDL1 positive GCs [4].

To explore the molecular mechanisms supporting tumour growth and its responsiveness to medical treatment, animal models are very helpful. "At the moment, the best preclinical model to validate targets and positive/negative predictors of response to therapy is represented by Patient-Derived

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✉ Rossella Reddavid
rossella.reddavid@unito.it

✉ Maurizio Degiuli
maurizio.degiuli@unito.it

Simona Corso
simona.corso@unito.it

Daniel Moya-Rull
daniel.moyarull@ircc.it

Silvia Giordano
silvia.giordano@unito.it

[1] Department of Oncology, Division of Surgical Oncology and Digestive Surgery, San Luigi University Hospital, University of Turin, Regione Gonzole 10, 10043 Orbassano, TO, Italy

[2] Department of Oncology, University of Torino, Candiolo, Italy

[3] Candiolo Cancer Institute, FPO-IRCCS, Candiolo, Italy

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Xenografts (PDXs), an experimental model that retains the principal histologic and genetic characteristics of the donor tumour, is predictive of clinical outcome, and is a valuable tool for personalized medicine decisions" [5]. This model summarizes many of the disease hallmarks of cancer patients, and is increasingly being applied to investigate existing and new drug therapies, tumour growth, and mechanisms of drug resistance. PDXs are usually generated by transplantation of human tumour tissue or cells into seriously immunodeficient mouse host strains. "Tumours that successfully engraft are further passaged to generate cohorts of tumour-bearing mice for experimental studies. PDX models are generated and used by researchers in academic, clinical, and pharmaceutical industry settings as well as specialized commercial organizations" [6].

Human subcutaneous tumour xenografts, grown in immunodeficient nude mice, morphologically, biologically, and biochemically closely resemble the original tumours [7–9].

The major problem of PDXs generated by subcutaneous implant is that the transplanted tumours are located in an abnormal microenvironment. Most subcutaneously implanted tumours are encircled by a pseudocapsule; having little chance to spread to the surrounding tissues seldom metastasize [10–12], regardless of their origin from highly aggressive tumours [13]. However, human tumours implanted orthotopically (that is, in the organs of origin of the tumour) in nude mice (Patient-Derived Orthotopic Xenograft—PDOX) show increased metastatic capability [13–17]. Therefore, human gastrointestinal tumours, orthotopically transplanted in athymic mice, can contribute to enhance our knowledge concerning cancer spreading growth and metastasis.

Recently, the technique of orthotopic xenograft has been ameliorated, from the "sewing" method to the "adhering" method [18–21]. The progress made in the operative technique strongly decreases the procedure duration and improves animals' morbidity and mortality. However, to date, an optimal GC animal model of orthotopic implantation employing intact tumour tissues is not yet well established.

In this review, we report the techniques for generating PDOX models so far described in the literature and we objectively evaluate the validity and faithfulness of these animal models as a reliable platform for preclinical experimental medicine in gastric cancer [6].

Survey methodology

Literature search and systematic review were done adhering to the Cochrane Collaboration guidance [22] to reduce the risk of bias and error. This review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement [23].

Literature searches

The research was conducted to identify studies of GC PDOX models, without any limitations by publication date, language, or publication status (published or unpublished). Search strategies are reported in Table 1. The following databases were investigated on 28 December 2018: Embase 1991_27/12/18, PubMed 1981_27/12/18, and Web of Science 1992_27/12/18 [24].

The searches were performed by the authors with the support of the Federate Library of Medicine of Turin.

Keywords were matched and included: in PubMed ("Stomach Neoplasms"[Mesh] OR ((gastric* OR stomach*) AND (neoplas* OR cancer OR cancers OR cancero* OR tumour* OR OR tumors OR tumora* OR malignan* OR

Table 1 Search strategies

Database	Research strategy	Studies matched
PubMed	("Stomach Neoplasms"[Mesh] OR ((gastric* OR stomach*) AND (neoplas* OR cancer OR cancers OR cancero* OR tumour* OR tumor OR tumors OR tumora* OR malignan* OR carcinoma*))) AND orthotopic* AND ("Mice"[Mesh] OR "Rats"[Mesh] OR "Animals"[Mesh] OR rat OR rats OR mouse OR mice OR murine* OR monkey* OR pig OR pigs OR porcine* OR animal* OR preclinical* OR pre-clinical* OR xenograft* OR xeno-graft* OR "Xenograft Model Antitumor Assays"[Mesh] OR PDOX)	313
Embase	('stomach cancer'/exp OR ((gastric* OR stomach*) AND (neoplas* OR cancer OR cancers OR cancero* OR tumour* OR tumor OR tumors OR tumora* OR malignan* OR carcinoma*))) AND ('orthotopic transplantation'/exp OR orthotopic*) AND ('mouse'/exp OR 'rat'/exp OR 'animal'/exp OR rat OR rats OR mouse OR mice OR murine* OR monkey* OR pig OR pigs OR porcine* OR animal* OR preclinical* OR pre-clinical* OR xenograft* OR xeno-graft* OR PDOX)	510
Web of science	(((gastric* OR stomach*) AND (neoplas* OR cancer OR cancers OR cancero* OR tumour* OR tumor OR tumors OR tumora* OR malignan* OR carcinoma*))) AND orthotopic* AND (rat OR rats OR mouse OR mice OR murine* OR monkey* OR pig OR pigs OR porcine* OR animal* OR preclinical* OR pre-clinical* OR xenograft* OR xeno-graft* OR PDOX)	319
Total		1142

carcinoma*))) AND orthotopic* AND ("Mice"[Mesh] OR "Rats"[Mesh] OR "Animals"[Mesh] OR rat OR rats OR mouse OR mice OR murine* OR monkey* OR pig OR pigs OR porcine* OR animal* OR preclinical* OR pre-clinical* OR xenograft* OR xeno-graft* OR "Xenograft Model Anti Assays"[Mesh] OR PDOX), in Embase ('stomach cancer'/exp OR ((gastric* OR stomach*) AND (neoplas* OR cancer OR cancers OR cancero* OR tumour* OR tumor OR tumors OR tumora* OR malignan* OR carcinoma*))) AND ('orthotopic transplantation'/exp OR orthotopic*) AND ('mouse'/exp OR 'rat'/exp OR 'animal'/exp OR rat OR rats OR mouse OR mice OR murine* OR monkey* OR pig OR pigs OR porcine* OR animal* OR preclinical* OR pre-clinical* OR xenograft* OR xeno-graft* OR PDOX) and in Web of Science (((gastric* OR stomach*) AND (neoplas* OR cancer OR cancers OR cancero* OR tumour* OR tumor OR tumors OR tumora* OR malignan* OR carcinoma*))) AND orthotopic* AND (rat OR rats OR mouse OR mice OR murine* OR monkey* OR pig OR pigs OR porcine* OR animal* OR preclinical* OR pre-clinical* OR xenograft* OR xeno-graft* OR PDOX).

Inclusion and exclusion criteria

Inclusion and exclusion criteria are detailed in Table 2. All original reports which were described in detail and validated the techniques to generate PDOX mouse models of human gastric cancer were included. Specifically, both the techniques of implantation of human cancer tissue fragments and cell cultures (≤ 3 passages) were searched.

Xenografts generated from metastatic tissue, cell lines, and those established in animals were excluded. Human cells genetically manipulated before implantation were not included, as well.

Non-English language papers, meeting proceedings, abstracts, letters to the editor, and commentaries were also excluded.

Study selection, data extraction, and data synthesis

Four independent reviewers screened titles and abstracts identified by literature search. Abstracts meeting all the inclusion criteria were obtained as full papers and were assessed to confirm whether also the full papers totally met these criteria. All studies excluded at this second step of the screening process were documented along with the reasons for exclusion. Any discrepancies among reviewers were solved through consensus. Data extraction was performed by three reviewers (AR, SR, and SS) and checked by a fourth reviewer (RR). Selected papers were identified by publication year and by the surname of the first Author.

Quality assessment

Study quality and model validity were assessed using SYRCLE's risk of bias tool for animal studies and Collins' certify tool adapted for PDOX [25, 26], respectively (Table 3a, 3b). Authors followed the ARRIVE guidelines checklist to evaluate the adequacy of each study for reporting animal research [27].

The study quality of included studies was independently assessed by four reviewers and any contrarieties were solved through discussion and consensus among them.

Results

Literature searches and inclusion assessment

Figure 1 summarizes the process of identification and selection of papers for inclusion in this systematic review, following the PRISMA guidelines [23].

Literature searches of electronic databases identified 1142 papers and hand searching retrieved 1 additional article. After de-duplication, 692 titles/abstracts were screened by reviewers and 404 articles were excluded as having no pertinence to this systematic review. Titles/abstracts of 288 potentially relevant papers were included for further evaluation and 245 of these were successively excluded, because they did not report technical details of PDOX generation. Of these, 33 papers were left out after examining in detail the full paper; the reasons for rejection are detailed in Fig. 1.

Overall, we identified ten studies of PDOX model validation properly fulfilling inclusion and exclusion criteria.

Table 2 Inclusion/exclusion criteria

Inclusion criteria	Exclusion criteria
Studies about surgical technique in creation of PDOX	No surgical technique description
Human cells sample from gastric cancer	Origin of human cells sample other than gastric cancer
Studies published in English	Previous genetic manipulation of human cells
	Only conference proceeding and abstract available online
	Studies published in other languages

PDOX Patient-Derived Orthotopic Xenograft

Table 3 **a** Study quality tool (SYRCLE's risk of bias tool for animal studies). **b** Model validity tool (Collins' certify tool adapted for PDOX)

	Signaling question	Project-specific notes	Decision	Justification
A				
Reporting				
1	Ethical statement specified was an ethical statement employed for animal manipulation?	Select reported/Not reported if the experimental protocols were approved by an ethical committee	Reported/ not reported	Justification comment
Validation				
2	Clear description of mouse model	Provide details of mouse characteristics—provenance, species, age, weight, strain. Select partially reported if only one is reported	Reported/Partially reported reported/Not reported	Justification comment
3	Clear description of the routine maintenance of the mice before and after transplantation	Provide details for mice feeding, isolation from other mice, temperature housing. Select partially reported if only one is reported	Reported/Partially reported reported/Not reported	Justification comment
4	Clear description of the preoperative care routine of the model before the experiment	Provide details for preoperative fasting, hair removal, antibiotics and/or analgesic administration. Select partially reported if only one is reported	Reported/Partially reported reported/Not reported	Justification comment
5	Clear description of the post-operative care routine of the model after the experiment	Provide details for post-operative fasting, antibiotics and/or analgesic administration. Select partially reported if only one is reported	Reported/Partially reported reported/Not reported	Justification comment
6	Clear description of the operative care routine	Provide details of anesthetic, oxygen administration, hydration, Select partially reported if only one is reported	Reported/Partially reported reported/Not reported	Justification comment
7	Clear description of the model euthanasia deadline	Provide details, indication and or timing for model euthanasia deadline. Select partially reported if only one is reported	Reported/Partially reported reported/Not reported	Justification comment
8	Clear description of the orthotropic tumour implantation technique	Provide details of the abdominal wall incision, implantation tumour size and place, suturing or fixing technique on the gastric wall Select partially reported if only one is reported	Reported/Partially reported reported/Not reported	Justification comment
9	Clear description of source specimen before orthotopic transplantation	Provide details of subcutaneous passages, injected gastric cancer cells or fresh specimen orthotopic transplantation	Reported/Not reported	Justification comment
A	Outcomes	Provide details of tumour local growth	%/Not reported	
B	Outcomes	Provide details of tumour metastasis site	% Site/Not reported	
Risk	Overall rating/reporting of model	*Low* all domains clearly reported, and there are no concerns with model. *Unclear* some domains are unclear, but not high risk. *High risk* there is a concern of high risk		Text to justify why model was given unclear or high risk

Table 3 (continued)

ID Study	Question 1	Question 2	Question 3	Question 4	Question 5	Question 6	Question 7	Question 8	Question 9	A	B	Overall rating/reporting of model	
B													
Furukawa [26]	Not reported	Partially reported	Partially reported	Not reported	Not reported	Partially reported	Reported	Reported	Reported	100% (intact tissue) 50% (cell suspension)	69.2% (intact tissue) 0% (cell suspension)	High	No ethical statement Unclear description of mice characteristics No description of perioperative care Unclear description of intraoperative protocol
Cui [27]	Not reported	Partially reported	Not reported	Not reported	Not reported	Partially reported	Reported	Reported	Reported	12.5%	0%	High	No ethical statement Unclear description of mice characteristics No description of peri operative care Unclear description of intraoperative protocol Low growth rate and metastases never occurred
Illert [28]	Reported	Reported	Reported	Not reported	Not reported	Partially reported	Reported	Partially reported	Reported	90%	70% Liver 10% Lung 10% Lymph node	High	Unclear description of mice characteristics No description of peri operative care Unclear description of intraoperative protocol
Illert [29]	Not reported	Reported	Reported	Not reported	Not reported	Partially reported	Reported	Partially reported	Reported	22% Fresh specimen 100% Cellular lines	11% Fresh specimen 88% Cellular lines	High	No ethical statement Unclear description of mice characteristics No description of peri operative care Unclear description of intraoperative protocol

Table 3 (continued)

ID Study	Question 1	Question 2	Question 3	Question 4	Question 5	Question 6	Question 7	Question 8	Question 9	A	B	Overall rating/reporting of model	
Jones-Bolin [30]	Reported	Reported	Partially reported	Not reported	Not reported	Reported	Reported	Reported	Reported	> 90%	40% Liver or lymph nodes 60% Peritoneal surface involving other organs, such as kidney, spleen, or diaphragm	U/NR	Not all validation criteria were met
Bhargava [18]	Not reported	Reported	Reported	Not reported	Partially reported	Reported	Reported	Reported	Reported	100%	33% Liver, lung, pancreas, retroperitoneum, kidney, bowel	U/NR	Not all validation criteria were met
Li [31]	Reported	Reported	Reported	Not reported	Not reported	Partially reported	Reported	Reported	Reported	100%	lymph nodes 58%, Liver 78%, kidney 39%, and peritoneum, diaphragm 81%	U/NR	Not all validation criteria were met
Li [32]	Reported	Reported	Reported	Not reported	Partially reported	Reported	Reported	Reported	Reported	100%	Lymph node 79%; liver 91%; kidney 62%; lung 25%; spleen 29%; testicle 20%; peritoneum 91%	Low	
Busuttil [33]	Reported	Partially reported	Reported	Not reported	Not reported	Reported	Reported	Reported	Reported	100%	80% Thoracic or abdominal	U/NR	Not all validation criteria were met
Feng [34]	Reported	Reported	Reported	Not reported	Not reported	Reported	Reported	Reported	Reported	100%	Liver, spleen, lung (20%)	U/NR	Not all validation criteria were met

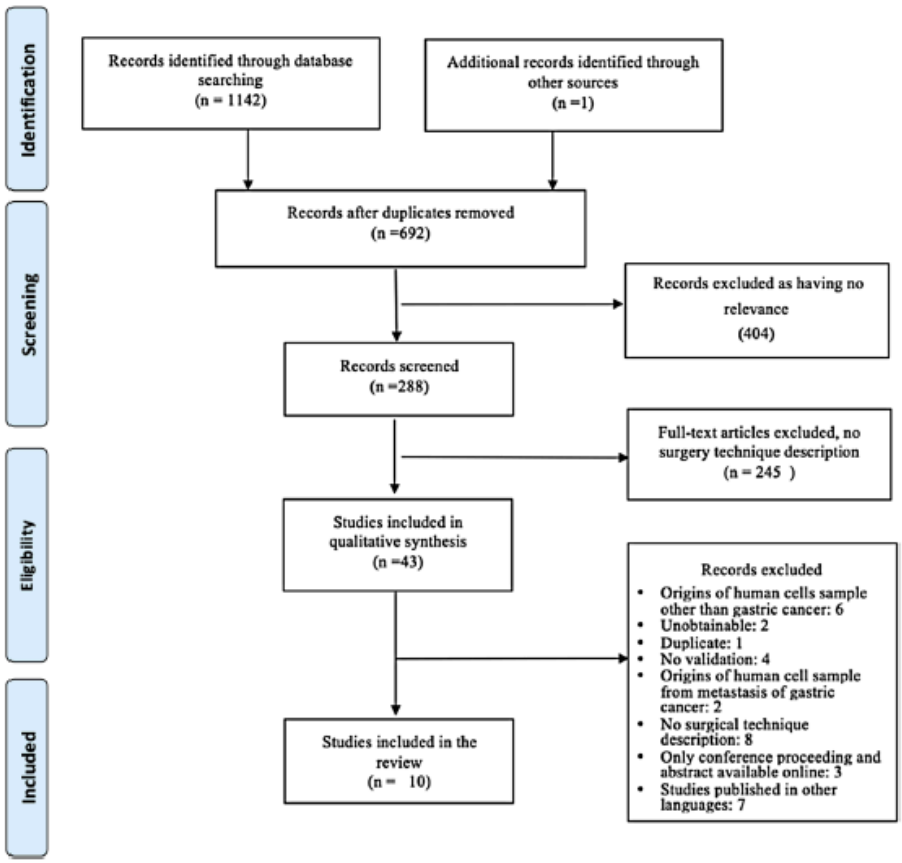

Fig. 1 PRISMA flow diagram of the study selection process

Table 4 provides a list of the included studies and their adherence to the ARRIVE guidelines.

Characteristics of PDOX models

Table 5a and b summarizes the main features of the PDOX models as described in the included studies.

The majority of these studies reported the use of a PDOX model to improve the understanding of carcinogenesis, cancer spread, and metastasis, and to support the research and the development of new and effective therapeutic concepts.

Several mouse strains were employed for derivation of gastric cancer animal models; the most common was Balb/c nu/nu (6 studies), athymic nude (2 studies), B16/Rag2/GammaC double knockout (1 study), and CD-1 nude (1 study) mice.

The most used site of engraftment for the derivation of gastric models was subcutaneous tissue (60%), followed by the gastric wall. Most models were derived from the engraftment of gastric cancer cell lines (70%), rather than tissue fragments.

Characteristics of transplantation technique

In 1993, Furukawa et al. [28] performed both orthotopic tumour tissue and cell suspension implantations (SC-1-NU from Nagoya University, H–1 11 from Osaka University, and St-4 and St-40 from the Central Institute for Experimental Animals Kawasaki lines). They reported two different techniques of orthotopic transplantation of *tissue fragments*. In the first approach, "an incision was made through the left upper abdominal pararectal line and peritoneum of the mice. The stomach wall was carefully exposed, and a part of the serosal membrane, about 3 mm in diameter in the middle of the greater curvature of the glandular stomach, was mechanically injured using scissors. A tumour piece of 150 mg was then fixed on each injured site of the serosal surface with a 4–0 Dexon transmural suture. In the other orthotopic tissue transplantation method, multiple tumour pieces were implanted on the top of the stomach where the serosa had been injured. An 8–0 surgical suture was used to penetrate these small tumour pieces and to suture them on the wall of the stomach" [28].

In a further method described by the same author in this report, *a tumour cell suspension* (0.1 ml per mouse)

Table 4 List of included studies and records and their adherence to ARRIVE guidelines

First author (year)	Article title	Primary research location	Country	Adherence to arrive guidelines
Furukawa [26]	Orthotopic transplantation of histologically intact clinical specimens of stomach cancer to nude mice: correlation of metastatic sites in mouse and individual patient donors	Department of Surgery, School of Medicine, Keio University. Tokyo, Japan	Japan	No
Cui [27]	Intact tissue of gastrointestinal cancer specimen orthotopically transplanted into nude mice	Clinic for General Surgery and Thoracic Surgery, and Institute of Pathology, Christian- Albrechts-University, Kiel, Germany	Germany	No
Illert [28]	Detection of disseminated tumor cells in nude mice with human gastric cancer	Department of Surgery, University Hospital, University of Würzburg, Germany;	Germany	No
Illert [29]	Optimization of a metastasizing human gastric cancer model in nude mice	Department of Surgery, University of Würzburg, Würzburg, Germany	Germany	No
Jones-Bolin [30]	Orthotopic models of human gastric carcinoma in nude mice: applications for study of tumor growth and progression	Oncology Research, Cephalon, Inc., West Chester, Pennsylvania	USA	No
Bhargava [18]	An orthotopic nude mouse model for preclinical research of gastric cardia cancer	Department of Surgery, Charité School of Medicine Campus Benjamin Franklin, Hindenburgdamm 30, 12203 Berlin, Germany	Germany	No
Li [31]	Serial observations on an orthotopic gastric cancer model constructed using improved implantation technique	Yan Li, Xiao-Ling Wu, Department of Gastroenterology, the Second Affiliated Hospital, Chongqing Medical University, Chongqing 400010, China	China	No
Li [32]	Characterization of gastric cancer models from different cell lines orthotopically constructed using improved implantation techniques	Yan Li, Xiao-Ling Wu, Department of Gastroenterology, the Second Affiliated Hospital, Chongqing Medical University, Chongqing 400010, China	China	No
Busuttil [33]	An orthotopic mouse model of gastric cancer invasion and metastasis	Upper Gastrointestinal Translational Research Laboratory, Peter MacCallum Cancer Centre, Parkville, VIC, Australia	Australia	No
Feng [34]	Characterization of an orthotopic gastric cancer mouse model with lymph node and organ metastases using bioluminescence imaging	Department of General Surgery and Hongqiao International Institute of Medicine, Shanghai Tongren Hospital	China	No

Table 5 **a** Animal features as reported in the included studies. **b** Surgical technique and results described in the included studies

ID Study	Animal features				
	Animal/tumour specimen	Animal/age (weeks)/sex/ number	Weight	Animal sacrifice time and criteria	Subcutaneous passages (N)
A					
Furukawa [26]	BALB/c nu/nu mice, fresh surgical from advanced gastric cancer and cell line	5 weeks/M/not specified	Not specified	12 and 10 to 24 weeks after implantations of intact tissue and cell suspension respectively	0
Cui [27]	Athymic naval medical research institute NMRI nude mice, tumour specimens from gastric cancer (fresh or frozen)	8–12 weeks/ M-F/8	Not specified	7th–21st week after implantation	Each mouse receives simultaneous orthotopic and subcutaneous implantation
Illert [28]	BALB/c nu/nu mice; gastric cancer cell line	6–10 weeks/ F/10	13–16 g	Tumour volume > 10 mm or declined general conditions	4 before orthotopic transplantation (group I), direct subcutaneous implantation (group II)
Illert [29]	BALB/c nu/nu mice; Tumour specimen and gastric cancer cell line	6–10 weeks/F/18	13–16 g	Tumour volume > 10 mm or declined general conditions	4 subcutaneous passages for cellular line, direct implantation for fresh specimen
Jones-Bolin [30]	Nude mice athymic nu/nu, gastric cancer cell line	6–8 weeks, F/not specified	20–25 g	declined general conditions/severe pain/bleeding or infected wound or tumour	1 subcutaneous passage
Bhargava [18]	CD-1 nude mice, Three gastric cancer cellular lines	4 weeks/M/180	20–30 g	Four, eight and twelve weeks after transplantation	1 subcutaneous passage
Li [31]	Balb/c nu–nu mice, gastric cancer cell line	5–6 weeks/M/36	18–20 g	Groups of animals were sacrificed every 2 weeks	1 subcutaneous passage
Li [32]	Balb/c nu–nu mice, gastric cancer cell line	5–6 weeks/M/24	18–20 g	Not sacrificed	1 subcutaneous passage
Busuttil [33]	Bl6/Rag2/GammaC double knockout mice, three gastric cancer cell lines	Age not specified, M/F/63	Not specified	1, 2, 4, and 6 weeks after injection	0
Feng [34]	BALB/c nude mice, luciferase-expressing human gastric cancer cell line	4–5 weeks, F/100	20 g	3rd–10th week after implantation	0

Table 5 (continued)

ID study	Surgical technique					Results		
	Anesthesia	Abdominal incision	Implantation site	Tumour size implanted	Laparotomy closure	Histological assay	Local growth (*N*)	Metastasis/site
B								
Furukawa [26]	2.5% Avertin	Left upper abdominal pararectal line and peritoneum	Serosa surface, greater curvature of the antrum	-A tumour piece of 150 mg -Multiple tumour pieces-0.1 ml of tumour cell suspension	Dexon 6–0 suture	Straight correlation between behavior of tumour in transplanted mice and patients	26/26 intact tissue 15/30 cell suspension	18/26 intact tissue 0/15 cell suspension
Cui [27]	1.2% Ketamine and 0.16% xylasin solution	Midline of the abdomen	Serosa surface, greater curvature of the antrum	2 mm^3	6–0 absorbable suture	Resemble the original tumours morphologically and biologically	1/8 12.5	0/8
Illert [28]	Isoflurane inhalation	Left-sided upper abdominal incision	Fixed to subserosa with 9–0 non-absorbable suture	10–20 mm^3	Two layers of 6–0 non-absorbable suture	Histological examination revealed: -infiltrating growth to adjacent structures -lung and liver showed the typical structure of metastatic adenocarcinoma. Positivity for immunostaining with the human CK-8-specific monoclonal antibody CAM 5.2 Infiltration of blood vessels was also observed as a sign of haematogenous metastases	9/10 orthotopic	7/10 orthotopic/liver 1/10 orthotopic/lung 1/10 orthotopic/lymph node
Illert [29]	Isoflurane inhalation	Left-sided upper abdominal incision	Fixed to subserosa with 9–0 non-absorbable suture	2–3 mm in length	Two layers of 6–0 non-absorbable suture	Tumour specimens: low local invasivity and distant spreading-Cellular lines: infiltrating growth to adjacent structures. Histology of the liver and lung showed the typical structure of metastatic adenocarcinoma Immunostaining positive for the human CK-8-specific monoclonal antibody CAM 5.2 Blood-vessel infiltration as a sign of haematogenous metastasis was observed as well	2/9 fresh specimens 9/9 cellular lines	1/9 fresh specimens 8/9 cellular lines

Table 5 (continued)

ID study	Surgical technique					Results		
	Anesthesia	Abdominal incision	Implantation site	Tumour size implanted	Laparotomy closure	Histological assay	Local growth (*N*)	Metastasis/site
Jones-Bolin [30]	Intramuscular injection of 100 µl ketamine/xylazine mixture, and isoflurane inhalation	0.5-cm horizontal incision just to the left of midline and under the rib cage	Serosal surface	$2 \times 2\ mm^3$	Two-to-four surgical knots of 6.0 Vicryl suture, using. Close the skin with 2 or 3 skin wound clips	Not reported	Primary tumour growth in > 90%	Liver or lymph nodes (40%), and peritoneal surface (60%) involving other organs
Bhargava [18]	Isoflurane inhalation for subcutaneous implantation; intraperitoneal xylazihydrochloride and Esketaminhydrochloride	Midline of the abdomen	Into submucosa layer of the distal stomach and gastric cardia	$1\ mm^3$	Two layers of 4–0 absorbable suture	Implantation of intact tumour fragments yielded in a complete tumour take rate. Cardia site seems more aggressive behavior	180/180	60/180 (liver, lung, pancreas, retroperitoneum, kidney, bowel)
Li [31]	Sumi-anxin II (0.02 ml per animal)	Left-side upper abdominal	Under serosal coat	$1\ mm^3$	4–0 absorbable suture	Glandular differentiation and rich vascularity were present in tumour areas. The stomach tumour invaded the gastric wall following the disruption of the integrity of the mucous layer or muscularis mucosae Smear of cast-off cells from ascites confirmed the malignant cells from the primary adenocarcinoma	36/36	lymph nodes 58%, liver 78%, kidney 39%, and peritoneum, diaphragm 81%
Li [32]	Sumi-anxin II (0.02 ml per animal)	Left-side upper abdominal	Under serosal coat	$1\ mm^3$	4–0 absorbable sutures	The stomach cancer of the two models infiltrated the various layers of gastric wall with disruption of the integrity of the mucous layer or muscularis mucosae	24/24	Lymph node 19/24; liver 22/24; kidney 15/24; lung 6/24; spleen 7/24; testicle 5/24; peritoneum 22/24
Busuttil [33]	Intraperitoneal Ketamine and Xylazine	Midline of the abdomen	Into subserous layer of the antral region	0.5 × 106 cells were resuspended in 50 ul Matrigel	4–0 and 3–0 Vicryl sutures	Not reported	Ranged from 72.4 to 82% based on cell line malignancy	80% thoracic or abdominal
Feng [34]	Not specified	Midline of abdomen	Subserosal layer	40 µl DMEM containing 5 × 106 NCI-N87-Luc cells	Not specified	Not reported	100/100	Liver, spleen, lung (20%)

was injected into the middle of the greater curvature of the exposed stomach. Mice were sacrificed 12 and 10–24 weeks after implantations of intact tissue fragments and cell suspension respectively, to evaluate tumour growth and metastasis. Both the rate of tumour engraftment and that of metastasis development were 100% after orthotopic implantation of intact human GC tissue fragments. On the contrary, they were reduced to 50% and 6.7%, respectively, after injection of cell suspensions. The authors concluded emphasizing the importance of implanting intact original tissue fragments to replicate the metastatic behavior of the cancer.

In 1998, Cui et al. [29] implanted surgical tumour specimens of 2 mm^3 on the serosal surface of the greater curvature of the antrum and fixed them with 6–0 absorbable transmural suture without subcutaneous passages. Mice were sacrificed at 7–21 weeks after tumour transplantation, and at autopsy gastric tumours, enlarged lymph nodes, lungs, and livers were removed and addressed to routine histological examination. Tumour growth was reported in 12.5% of cases and metastases never occurred.

Illert et al. published two articles in 2003 about orthotopic transplantation of human GC in nude mouse [30, 31]. In the first article [30], two groups of nude mice were used for xenotransplantation of GC specimens. In group I, tumour specimens originating from the gastric adenocarcinoma cell line 23,132/87 (DSMZ Braunschweig, Germany) were transplanted onto the stomach; in group II, they were transplanted subcutaneously into both axillaries. Animals of group I were operated on via a left-sided upper abdominal incision. "The stomach was exteriorized and the serosa of the anterior wall was removed with a scalpel. Two or three tumour cubes of approximately 10–20 mm^3 were sewn on the prepared gastric wall with 9.0 non-absorbable sutures. Animals were sacrificed if the tumour showed a growth of 10 mm diameter or if their general condition declined; frozen sections of tumours and organs (abdominal organs, lymph nodes, lungs, and retroperitoneal organs) were histologically examined" [30]. The authors reported primary tumour growth in 90% of mice and metastases spread to the liver (70%), lung (10%), and lymph nodes (10%).

In their second article, Illert et al. [31] performed orthotopic xenotransplantation of primary GCs (series 1) and of tumour fragments derived from the GC cell line 23,132/87 (DSMZ, Braunschweig, Germany) (series 2). "Animals were laparotomized via a left-sided upper abdominal incision. The stomach was exteriorized, and the serosa of the anterior wall was removed with a scalpel. Two or three tumour cubes of approximately 2–3 mm in length were sewn onto the exposed gastric wall with 9.0 non-absorbable sutures, using microsurgical techniques. Animals were sacrificed after tumour growth reached 10 mm in diameter, or if tumour growth induced a general health decline in mice. All animals were dissected and examined macroscopically" [31]. Tumour growth was poor and slow in series 1 (22% growth rate), while all mice developed tumours in series 2 (100% growth rate) and distant metastases occurred in 11% and 88% in series 1 and 2, respectively.

In Jones-Bolin's trial [32], human GC xenograft tumours were generated by injecting human GTL-16 GC cells subcutaneously. The reported orthotopic transplantation technique is characterized by the following steps: a small horizontal skin incision was made over the left-lateral abdominal area; once the underside of the stomach was exposed, two 2×2 mm^3 tumour fragments were pierced with a needle of 6/0 prolene and gently glided onto the prolene wire. Tissue fragments were sewn to the dorsal side of the stomach in the mid-section, using two or three knots. Mice were sacrificed when they lost > 15% body weight, and developed ascites or after 8 weeks from implantation. At necropsy, tumour spread throughout the peritoneum and to different organ sites was grossly assessed. The authors reported primary tumour growth in > 90% of mice and metastasis spread to the liver (40%), lymph nodes (40%), and peritoneal surface (60%) involving several organs, such as kidney, spleen, or diaphragm, in addition to the development of ascites.

In Bhargava's report [19], three different human GC cell lines ("AGS poorly differentiated, MKN-45 poorly differentiated, and NCI-N87 well differentiated, from the European Collection of Cell Cultures Salisbury, UK") were injected subcutaneously to originate tumour fragments. "The animals' abdomens were opened by a midline incision and the stomach was gently exteriorized. One small tissue pocket was prepared either in the submucosa of the distal stomach or the gastric cardia using a microscissor. One donor tumour fragment was placed into each gastric tissue pocket and fixed with one drop of tissue glue (Hystoacryl, B. Braun, Tuttlingen, Germany)" [19]. Four, eight, and twelve weeks after implantation, ten animals of both distal stomach or gastric cardia implantation-site groups, and of each GC cell line group, were sacrificed and a necropsy was done to assess tumour spread. Implantation of donor tumour fragments resulted in orthotopic tumour growth in 100% of both implantation-site groups despite the type of cell line used for obtaining the tissue fragment. Metastatic spread to the lung, pancreas, liver, bowel, retroperitoneum, and kidney was reported from 10 to 50% after 12 weeks.

In Li's trial [33] human gastric cancer cell suspensions (SGC-7901 poorly differentiated, obtained from the Centre of Cell Cultures of Chinese Academy of Medical Sciences, Shanghai, China) were inoculated subcutaneously into a nude mouse to originate solid tumours, and fragments of these tumours were engrafted beneath the serosal layer. A small tissue pouch was made in the serosal layer in the middle of the greater curvature with the help of a microscissor. A tumour scrap was inserted into the small pouch and pasted with a spot of "medical tissue glue (Shunkang Corporation

of Biological Adhesive, Beijing, China)" [33]. Engrafted animals were progressively necropsied every 2 weeks to assess primary tumour growth, spread, and metastasization. Primary tumour was reported to develop from 2 to 4 weeks, gradually increase in volume starting from the 6th week after implantation, and reach a growth peak at the 12th week. The rate and sites of metastasis were analyzed and "the following results were reported: lymph nodes 58%, liver 78%, kidney 39%, and peritoneum and diaphragm 81%" [33].

Li et al. [34] published a second article on orthotopic transplantation. Two different human GC cell suspensions ("SGC-7901 and BGC-823 purchased from the Centre of Cell Cultures of Chinese Academy of Medical Sciences, Shanghai, China") were injected subcutaneously to originate solid tumours, and then, tumour fragments were implanted under the serosal coat of the stomach. A left-lateral upper abdominal incision was made, the stomach was exteriorized, a small tissue pouch was done in the middle of the greater curvature with the help of a microscissor, and then, a tumour scrap was pasted into the tissue pouch with a spot of medical tissue adhesive (Shunkang corporation of Biological Adhesive, Beijing, China). At animals' death, mice were necropsied to assess tumour growth and metastases. The tumour uptake rate after orthotopic implantation in both groups was 100%. The authors observed high incidence of metastases in lymph nodes (79%), liver (91.5%), kidney (62.5%), lung (25%), peritoneum or diaphragm (91%), spleen (58%), and testis (42%).

In Busuttil's trial [35], the authors tagged three different gastric cancer cell lines (MKN45, AGS, and MKN28 from Murdoch Children's Research Institute) with luciferase; tagged cell lines were then inoculated into the subserosal coat of the stomach. In their surgical technique, "a subxiphoid midline incision was made and the stomach was exteriorised. A dissecting microscope was used to guide the needle containing 50 µl of the cell/Matrigel suspension into the subserosal layer of the antral region of the stomach" [35]. The injection was given very slowly to avoid leakages because of unnecessary pressure. "The needle was withdrawn after 20 s to allow the Matrigel to set and prevent inadvertent abdominal seeding of the tumour cells. Successful positioning of the transplant was confirmed by the presence of a Matrigel "bleb"" [35]. D-Luciferin (Xenogen) was then injected intraperitoneal and images were taken using IVIS Living Image 3.0 software. Engrafted tumour growth and spread were monitored by submitting mice to weekly diagnostic imaging. Animals were sacrificed at specific time frames and immediately autopsied. Soon after gross examination, the stomach and major organs were resected and submitted to imaging technique. Tumour uptake with GC cells ranged from 72.4 to 82% based on cell line aggressiveness. A high rate of metastasis was reported in multiple sites within abdominal and thoracic regions.

In the last trial, Feng et al. [36] injected luciferase-expressing NCI-N87 human GC cells, obtained from the Shanghai Biomodel Organisms Center Co., Ltd. (Shanghai, China), into the subserosa of the gastric body. The orthotopic technique was performed opening the mouse abdomen via a midline incision and exteriorising the stomach. Cancer cells were inoculated into the subserosa of the middle of the stomach using a 100 µl syringe with 30G needle. A cotton swab was pressed against the injection site for ≥ 20 s to prevent tumour cell leakage into the peritoneal cavity. At 4, 6, 8, and 10 weeks after tumour cells injection, a 200 µl solution of D-luciferin was intraperitoneally injected. "Subsequently, mice were anesthetized and at 8 min after D-luciferin injection, placed in the Xenogen IVIS 200 chamber with right lateral recumbency for bioluminescence imaging of the orthotopic NCI-N87-Luc tumour" [36]. To assess tumor spread in major organs and in lymph nodes at several time frames, mice were culled between week 3 and 10. Lymph nodes, kidney, lung, liver, spleen, and the heart were removed and processed for ex vivo *BLI* (bioluminescence imaging) and histopathological examination. The rate of tumour engraftment in the stomach was 100%. On the opposite, metastases were observed only in a few cases and the authors concluded that unsuccessful metastatic spread could be referred to the well-differentiated characterization of NCI-N87 cell line.

Model validity

The model validity tool formerly described by Collins et al. [26] was modified to include further advices specifically for the PDOX models (Table 3a, b). Not even a single study reported that the ARRIVE guidelines or SYRCLE's risk of bias tool [25, 27] had been followed.

Discussion

The failure of actual animal models to definitely predict anticancer activity of investigated therapies also in the clinic represents one of the most serious obstacles confronting investigators involved in drug development. On the contrary, PDOX models are thought to be useful to assess the efficacy of single drugs (or their combination) targeting molecular lesions recognized in the engrafted tumours. As a matter of fact, orthotopic implantation of human tumour better mimics the original microenvironment of the tumour itself, and thus, the therapeutic responses to therapies observed in patients can be more easily reproduced.

The strain, sex, and age of mice employed, site and type of tumour engraftment (cell line or tissue fragment), timing of donor autopsy, technique of in vivo or ex vivo imaging to detect tumour growth and metastases, and rate of engraftment and metastatic spread were investigated in this

review. The quality of the studies was analyzed according to a modified validity tool proposed by Collins et al. [26], and to the guidelines of animal care and use. The provision of the approval of the Ethical Committee was also enquired.

An important issue in the generation of PDXs and PDOXs is the choice of the mouse strain. In fact, it has been shown that the degree of immunodeficiency affect the engraftment rate, as the most immunodeficient strains usually show the highest take rate; however, they are also more frequently affected by immunoproliferative disease and much more expensive. The majority of Authors (6 of 10) used Balb/c nu–nu mice, while more severely immunodeficient mice strains like the NMRI nude mice, B16/Rag2/GammaC double knockout, or CD-1 mice were employed as tumour hosts in the other reports to improve the engraftment and metastatic spread rates. Nevertheless, despite the use of NMRI athymic nude mice (a specific strain of mice with reduced natural killer cell activity), the reported tumour take rate was very low (7%) and metastases never occurred [29].

The age and sex of mice were different among the studies, but they did not affect post-operative complications or mortality. These results do not reflect data from the literature that identify the adult and male mice as stronger.

Most models were derived from the engraftment of established human gastric cancer cell lines (70%), rather than tissue fragments; in two papers, the authors performed both orthotopic tumour tissue pieces and cell suspension implantations. The use of primary gastric cancer cell lines rather than established ones could be more interesting as it is known that long-term culture can allow the onset of many genetic alterations that can affect tumour behavior and metastatic ability.

Animals were sacrificed following different timing criteria; in three studies, they were euthanized when they developed distress signs or when cancer size reached more than 10 mm; in the other articles, they were sacrificed systematically to evidence progressive tumour growth and metastases appearance. Finally, in Li's study [34], mice were submitted to autopsy soon after their natural death.

In Busuttil's and Feng's [35, 36] articles, the IVIS technology was employed after D-luciferin injection to obtain in vivo and ex vivo bioluminescence imaging. No other in vivo or ex vivo imaging procedures were performed in the examined articles.

In 90% of studies, tumour tissue fragments or cell suspensions were, respectively, fixed or injected into the subserosal layer. In only one study, tumour fragments deriving from a cell line were implanted into the submucosal layer [19]. Considering that human gastric cancer originates from glandular cells located in the mucosal layer, theoretically, an adequate PDOX model with implantation of tissue/cells in the proper layer still does not exist; only one out of ten studies examined reported a model close to the optimal one. In practice, no significant difference of tumour engraftment and metastatic spread rates was observed by authors reporting models with either submucosal or subserosal implantation of tumour tissue/cells.

There was ambiguity in the author's definition of 'successful' primary tumour growth; success was based sometimes on a specific tumour size and sometimes on tumour growth after a specific time interval.

Although PDOXs are claimed to better mimic donor tumours, as they are also able to develop metastases, and could be used to validate new molecular targeting therapies also for patients with stage IV cancer, their metastatic spread rate was not homogeneous in the examined studies. While it was not observed in one study (implantation of original tumour tissue onto the serosal surface), it was, however, observed at high rates in 40% of PDOX models employing either cell suspension injection or cell-line-derived tissue fragments (1 passage s.c.), both into the subserosal layer [29, 32–34].

More than half of the studies (60%) provided ethical statements and followed guidelines for the use of animal and human tissue. It was not the proper goal of this systematic review to strictly evaluate whether authors of included papers reported data in accordance with the guidelines of animal care and use, but it is remarkable that an ethical statement was not provided in 40% of selected studies, and most of them did not even report a clear description of the routine maintenance of animals before and/or after procedure.

In accordance with our modified model validity tool, half of the studies were classified as unclear, because at least one validation condition was not reported, or researchers did not provide proper data for a part of their models (Table 3b). The most frequent missing data were the details of perioperative management of mice and the accurate description of all steps of the surgical technique. Such information is absolutely necessary to enable other investigators to reproduce models and researches and verify formerly reported findings.

Another important point is that authors validated their models with a very small sample. The number of mice employed is not reported in two studies [28, 32] and is lower than ten in another two of the ten papers considered for this review [29, 30].

The model validity tool described here provides an 'ideal set of validation criteria' for PDOX animal models, and can be adjusted and applied to other models or studies (Table 3b) [24]. Providing evidence to respect all these criteria should be reasonable for a research group. The majority of the included studies described the use of PDOX models for gastric cancer preclinical research or drug development, emphasizing the importance of meticulous validation of these preclinical models.

Gastric cancer PDOX models so far available in literature are not properly adequate according to the model validity

tool derived from Collins' former proposal and were mostly validated with low samples [26]. So far, there is no demonstration that the submucosal (or mucosal) site is more effective than the serosal surface or the subserosal layer for tissue implantation or cell suspension injection, and that tissue fragment implantation is better than cell suspension injection. Importantly, there is only one report of successful orthotopic implantation of primary human tumour tissue [28].

Conclusions

Further studies on gastric cancer PDOX should strictly employ model validity tools and larger samples with orthotopic implantation sites mirroring as much as possible the donor tumour characteristics and microenvironment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. No financial support.

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent It was not necessary, because it is a systematic review.

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ORIGINAL ARTICLE

Personalized therapeutic strategies in HER2-driven gastric cancer

Stefano Ughetto[1,2] · Cristina Migliore[1,2] · Filippo Pietrantonio[3,4] · Maria Apicella[2] · Annalisa Petrelli[2] · Laura D'Errico[1,2] · Stefania Durando[2] · Daniel Moya-Rull[2] · Sara E. Bellomo[1,2] · Sabrina Rizzolio[2] · Tania Capelôa[2,16] · Salvatore Ribisi[2] · Maurizio Degiuli[5] · Rossella Reddavid[5] · Ida Rapa[5] · Uberto Fumagalli[6,17] · Stefano De Pascale[6,17] · Dario Ribero[2] · Carla Baronchelli[7] · Giovanni Sgroi[8] · Emanuele Rausa[8] · Gian Luca Baiocchi[9] · Sarah Molfino[9] · Stefania Manenti[7] · Maria Bencivenga[10] · Michele Sacco[10] · Claudia Castelli[18] · Salvatore Siena[4,11] · Andrea Sartore-Bianchi[4,11] · Federica Tosi[4,11] · Federica Morano[3] · Alessandra Raimondi[3] · Michele Prisciandaro[3,4] · Annunziata Gloghini[12] · Silvia Marsoni[13] · Antonino Sottile[2] · Ivana Sarotto[2] · Anna Sapino[2,14] · Caterina Marchiò[2,14] · Paola Cassoni[14] · Simonetta Guarrera[2,15] · Simona Corso[1,2] · Silvia Giordano[1,2]

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Abstract

Background Trastuzumab is the only approved targeted therapy in patients with *HER2*-amplified metastatic gastric cancer (GC). Regrettably, in clinical practice, only a fraction of them achieves long-term benefit from trastuzumab-based upfront strategy. To advance precision oncology, we investigated the therapeutic efficacy of different HER2-targeted strategies, in *HER2* "hyper"-amplified (≥ 8 copies) tumors.

Methods 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 (PDXs), through the design of ad hoc preclinical trials.

Results Despite the high level of *HER2* amplification, trastuzumab elicited a partial response only in 2 out of 8 PDX models. The dual-HER2 blockade with trastuzumab plus either pertuzumab or lapatinib led to complete and durable responses in 5 (62.5%) out of 8 models, including one tumor bearing a concomitant *HER2* mutation. In a resistant PDX harboring *KRAS* amplification, the novel antibody–drug conjugate trastuzumab deruxtecan (but not trastuzumab emtansine) 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.

Conclusion 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. Our results suggest that a selected subpopulation of *HER2*-"hyper"-amplified GC patients could strongly benefit from this strategy. Despite the negative results of clinical trials, the dual blockade should be reconsidered for patients with clearly HER2-addicted cancers.

Keywords HER2 · Targeted therapy · Trastuzumab · Gastric cancer · Drug resistance

Introduction

Trastuzumab is the only approved targeted therapy in patients with metastatic gastric cancer (mGC) bearing overexpression/amplification of *HER2*, a molecular driver belonging to the Epidermal Growth Factor Receptor (EGFR) family of receptor tyrosine kinases (RTKs). Constitutive activation of the HER2 pathway, usually due to gene amplification or mutations, has been observed in several solid tumors where it drives tumor growth, metastasis and

Stefano Ughetto, Cristina Migliore equally contributed to the work.

Simona Corso, Silvia Giordano equally contributed to the work.

✉ Silvia Giordano
silvia.giordano@unito.it

Extended author information available on the last page of the article

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angiogenesis [1]. *HER2* is amplified in around 10–15% of GCs [2, 3] and is usually associated with the "chromosomal instability" (CIN) molecular subtype, although elevated intra-tumor and inter-lesion heterogeneity has been found in *HER2*-amplified GCs [4, 5]. The post hoc analysis of the ToGA trial demonstrated that the addition of the monoclonal antibody (MoAb) trastuzumab to chemotherapy significantly improved overall survival (OS) in patients with locally advanced or metastatic gastric or gastro-esophageal junction cancers, but exclusively in the subgroup with higher levels of HER2 expression (IHC 3 + or IHC 2 + with gene amplification). Regretfully, only a fraction of patients with *HER2*-amplified mGC clearly benefits from trastuzumab, casting doubts on the actual cost-effectiveness of this regimen in clinical practice for the overall HER2-positive population. Indeed, the limitation of long-term efficacy of trastuzumab-based treatment may be due to the high percentage of primary and acquired resistance mechanisms involving *RTK/KRAS* co-amplifications, PI3K/Akt axis deregulation, *HER2* loss, heterogeneity of HER2 proteome including d16HER2 splice variants and, potentially, non-cell autonomous mechanisms [6–9].

Beyond trastuzumab, several HER2-targeted drugs are currently approved in patients with HER2-positive breast cancer, including the MoAb pertuzumab combined with trastuzumab, the small molecule EGFR/HER2 tyrosine kinase inhibitor lapatinib and the antibody–drug conjugates (ADC) trastuzumab emtansine (T-DM1) and trastuzumab deruxtecam (DS-8201a). However, the phase 3 trials conducted with these agents in mGC were all negative [10–12]. While in breast and gastric cancers, trastuzumab is effective also as monotherapy, in other cancers—such as colorectal cancer—only dual-HER2 blockade with trastuzumab plus other anti-HER2 drugs (including pertuzumab or lapatinib) has shown significant activity [13–15]. Notably, the level of *HER2* amplification may greatly impact the long-term efficacy of trastuzumab, with *HER2* gene copy number to chromosome 17 centromere ratio of 4.7 suggested as the optimal cut-off value to identify patients with exceptional response [16]. Based on the above assumptions, it is conceivable that the potential role of novel HER2-targeted strategies or dual anti-HER2 blockade should be re-assessed in molecularly selected patients with *HER2* "hyper"-amplified/HER2-addicted cancers.

At present, the optimal preclinical model to validate targets and identify effective treatments is represented by Patient-Derived Xenografts (PDXs), which combine the versatility of preclinical evaluation with the informative significance of population-based studies [17, 18]. Taking advantage of a proprietary, molecularly annotated colony of GC PDXs [19], we undertook a prospective evaluation of the therapeutic efficacy of different HER2-targeted strategies, in a selected subgroup of *HER2* "hyper"-amplified xeno-patients through the design of ad hoc preclinical trials aimed at improving personalized treatment of HER2-positive GC.

Materials and methods

Patients

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

Primary cell cultures

GC primary cells were derived from PDXs as described in [20]. The genetic identity of the in vitro-derived material with the original tumor has been verified by short tandem repeat profiling (Cell ID, Promega). For cell viability assays, cells were seeded in quadruplicate well in 96-well culture plates ($3–5 \times 10^3$ cells/well), in the presence of the indicated drugs. After 6 days, cell viability was measured using the Cell TiterGlo Luminescent Cell Viability Assay (Promega).

Western blot analysis

Cells were treated for 24 h 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).

EBV evaluation

Detection and quantification of EBV DNA were performed using the EBV Q-PCR Alert KIT (ELITechGroup S.p.A., Puteaux, France). The real-time amplification assay was carried out on ABI 7300 Real-Time PCR System instrument (Applied Biosystems, USA). PDXs were classified as described in [21]: 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.

PDX

Generation

Gastric PDX generation was performed as described in [19]. All animal procedures adhered to the 'Animal Research: Reporting of In Vivo Experiments' (ARRIVE) standards and were approved by the Ethical Committee of the Candiolo Cancer Institute, and by the Italian Ministry of Health.

Xenotrials

PDXs were passaged and expanded for > 2 generations until production of a cohort of mice. 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.

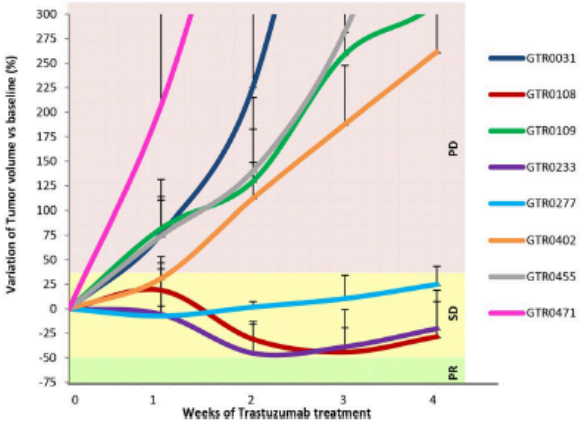


Fig. 1 Response to trastuzumab treatment in PDXs bearing high *HER2* CNG. The Spaghetti plot illustrates the effect of trastuzumab treatment (30 mg/kg) on PDXs with a *HER2* CNG ≥ 8 copies. Individual lines represent, for each PDX model, the mean percentage variation in tumor burden, from treatment start (day 0) to 4 weekly consecutive serial assessments (N = 5 mice for each model). Tumor response has been evaluated using RECIST 1.1-like criteria according to [22]: 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)

Response to treatment

The response in mice has been evaluated using RECIST 1.1-like criteria, i.e. progressive disease (PD): ≥ 35% increase from baseline; partial response (PR): ≥ 50% reduction from baseline; stable disease (SD): intermediate variations from baseline [22]. Statistical testing for pharmacological experiment was performed with GraphPAD PRISM Software 8.0, using Two-way ANOVA followed by Bonferroni multiple comparisons correction. Statistical significance: ns = not significant; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$.

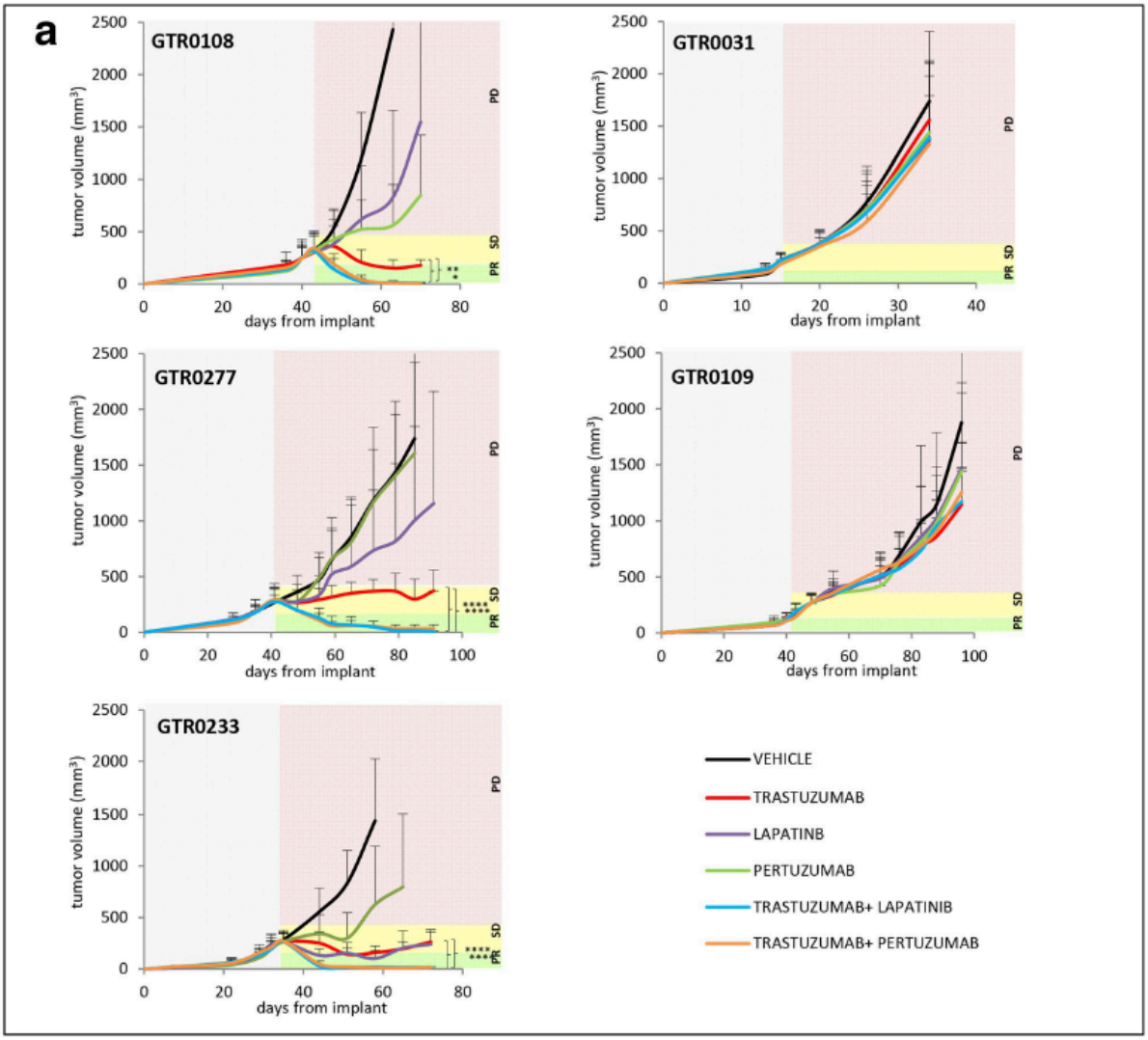
Genomic sequencing

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 (Macrogene Inc, Seoul, Korea).

In situ Hybridization and Immunohistochemistry

Dual-color FISH was performed on 4 µm thick sections using probes for *HER2* (17q12) and *CEP17* (Vysis, Inc, Downers Grove, IL, USA), as previously described [24]. IHC for HER2 was performed on 4 µm thick sections in a centralized manner at Candiolo Cancer Institute, using the HercepTest™per Dako Autostainer (Agilent). Immunohistochemistry for P-MET was performed using the P-MET (Tyr1234/1235) antibody AF2480 from R&D Systems.

The RNAscope probe for mouse HGF (Mm-Hgf-O1 #435381, Advanced Cell Diagnostics) was hybridized on 4 µm FFPE slides following the RNAscope 2.5RED assay protocol (#322452 and #322360). Sequential slides were stained with a mouse-specific control probe (mmPPIB: Peptidyl-prolyl *cis–trans* isomerase B, not shown). After that, the same slides have been hybridized, through immunohistochemistry experiment, with anti-Human cytokeratin antibody (CloneAE1/AE3 #M3515-DAKO Glostrup Denmark) following the standard protocol. To quantify the amount of positive signals in the stromal (pan-cytokeratin negative) areas, at least 2 digital images/slide have been captured at 20 × magnification. RNAscope-positive regions (red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GTR0108
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GTR0109
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tumor volume (mm³)
days from implant
PD
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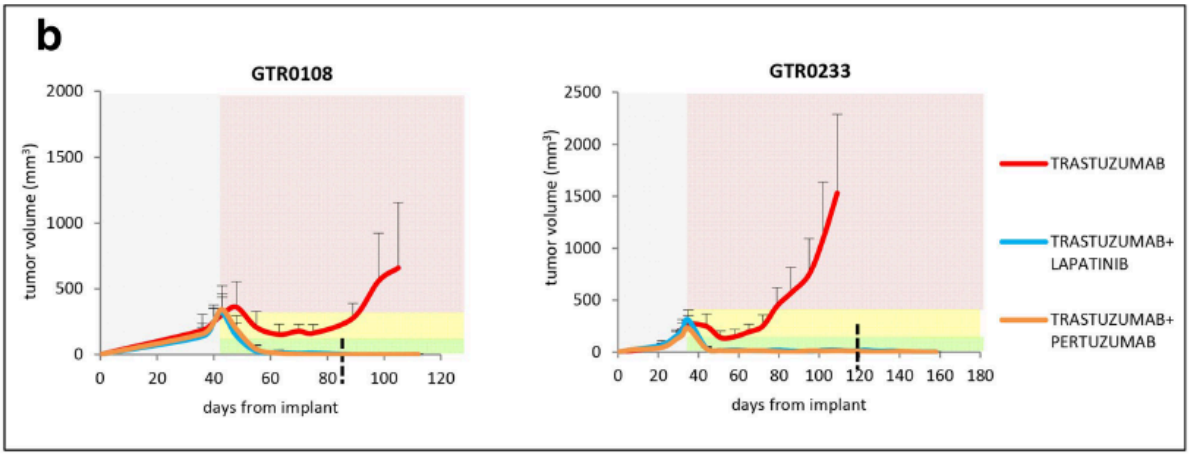
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◂**Fig. 2** Dual HER2 blockade is the most effective and durable treatment in *HER2*-amplified PDXs. **a** Tumor growth curves of mice cohorts derived from GTR0108, GTR0233, GTR0277, GTR0031 and GTR0109 patients, treated with the HER2 inhibitors trastuzumab, 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 background); partial response (PR): ≥ 50% reduction from baseline (green background); stable disease (SD): intermediate variations from baseline (yellow background). Complete Response (CR): 100% reduction from baseline. **b** Tumor growth curves of mice cohorts derived from GTR0108 and GTR0233 patients 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

hue regions) were quantified using ImageJ Software (NIH). Briefly, nuclei enclosed into tumor areas (pan-cytocheratin staining) have been excluded from the analysis; the area corresponding to stromal compartment has been computed; the background has been subtracted. Color deconvolution was performed using HPAS as vector, to split in three images, one for each channel (first channel contains nuclei, second channel RNAscope-positive dots). Finally, particle analysis was performed for the two channels by setting the same size and circularity for all the images in the same channel. Positivity for every analized images, as proxy of the amount of stromal HGF, was as follows: RNAscope-positive area/(nuclei area + RNAscope-positive area)*100. Analyses have been performed in blind.

Results

Prevalence of *HER2* amplification in gastric cancer PDXs and response to trastuzumab

Preclinical and clinical data obtained from tumors displaying *HER2* amplification have shown that the clinically relevant threshold is at least 8 gene copies [16]. Thus, we analyzed by real-time qPCR 570 primary GCs and identified primary tumors bearing ≥ 8 *HER2* gene copies (Suppl.Table 1). Eight of these patients were treated with a trastuzumab-containing therapeutic regimen: among them six experienced response to treatment (SD or PR).

From the 570 samples, we were able to generate PDXs in 151 cases. Among them, 8 PDXs were bearing ≥ 8 gene copies (Suppl. Table 1). FISH analysis confirmed *HER2* gene amplification and IHC analysis revealed that all the models were HER2 3 + (Suppl. Figure 1). The histopathologic features of the PDXs recapitulated those of the tumors of origin (Suppl. Figure 2A). Moreover, in the cases where it was possible to perform molecular analyses on the primary tumor, we observed a good concordance between the primary tumors and the corresponding PDXs relative to the mutational profiles, the methylation pattern of the top 1000 most variable CpG sites and *HER2* methylation pattern (Supplementary Fig. 2B, C, D).

These PDX models were passaged in vivo until five tumor-bearing animals/treatment group were produced, to evaluate the effect of the pure HER2 inhibition, without the confounding effect of chemotherapy. 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 (see Methods and Figure Legend). As shown in Fig. 1, 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).

Trastuzumab plus lapatinib or pertuzumab combinatorial therapies are more effective than trastuzumab monotherapy in *HER2* hyper-amplified GC PDXs

To evaluate whether dual-HER2 blockade may improve the efficacy in terms of response compared to trastuzumab monotherapy, we tested different HER2-targeted drugs or combinations in five HER2 + PDXs scoring 3 + at the IHC HercepTest and bearing ≥ 8 *HER2* copies. These tumors, at least in principle, have the maximal probability of being trastuzumab sensitive [6]. The different treatment groups were: (1) trastuzumab ("gold standard"); (2) pertuzumab (anti-HER2 MoAb mainly disrupting ligand-induced HER2 heterodimers); (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 Fig. 2a, 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 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, displaying around 300 *HER2* gene copies, we identified a *HER3* activating mutation (p.G284R) [25] that could be responsible for the relatively low sensitivity to trastuzumab monotherapy (achievement of SD, Suppl. Figure 3A). Indeed, the

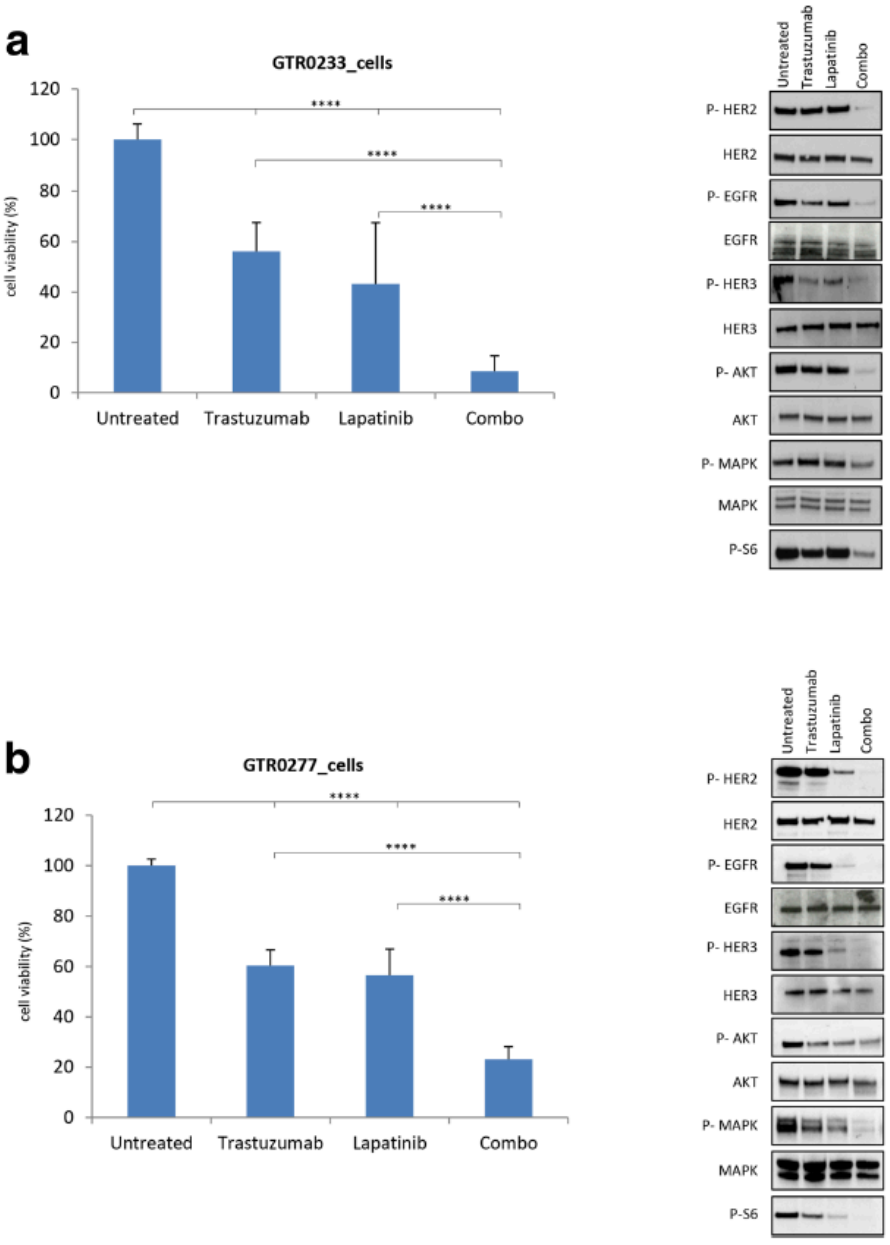


Fig. 3 Dual HER2 blockade is more effective than trastuzumab alone in GTR0233 and GTR0277 PDX-derived cells in vitro. Cell viability assay performed on GTR0233 (**a**, left panel) and GTR0277 (**b**, left panel) tumor-derived cells, upon treatment for 6 days with the indicated drugs at IC50 for each 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 h 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

dual-HER2 block, interfering with heterodimers formation and activation, led to a complete response (Fig. 2). From this PDX, we derived in vitro primary cells which maintained both *HER2* amplification and the *HER3* mutation (Suppl. Figure 3B, C). In vitro experiments showed that combinatorial treatment with trastuzumab plus an anti-HER3 MoAb (MM-121/seribantumab) resulted in a strong growth inhibition (Suppl. Figure 3D).

In 2 PDX models (GTR0108 and GTR0233), we performed long-term experiments to evaluate the possible onset of secondary resistance to the mono and combo treatments. As shown in Fig. 2b, 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).

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 (Suppl. Figure 3B and 4)] 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 (Fig. 3a, 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 (Fig. 3a, b, right part). Very similar results were obtained with organoids derived from the GTR0108 PDX (Suppl. Figure 5A–C). 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 combinations resulted in durable CRs.

In two cases (GTR0031, GTR0109, displaying 10 and 8 *HER2* copies, respectively), we did not observe any response to the investigated anti-HER2 strategies (Fig. 2a). Genomic analysis of the GTR0031 model revealed the presence of *KRAS* co-amplification (8 copies), shown to be responsible for resistance to RTK targeting in different tumor contexts [8, 26–29] (Suppl. Figure 6A). No putative genomic alteration likely sustaining trastuzumab resistance was identified in GTR0109.

As the HER2-targeting ADC T-DM1, consisting of the humanized MoAb trastuzumab covalently linked to the cytotoxic agent DM1, is effective in breast cancer, we investigated whether T-DM1 could overcome trastuzumab resistance in these two non-responsive PDXs. As shown in Suppl. Figure 6B, 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) has recently shown clinical activity in patients with advanced breast cancer after failure of all standard anti-HER2 agents including T-DM1 [30]. As DS-8201a has shown preliminary activity also in patients with heavily pre-treated HER2-positive mGC [31], we administered this agent to GTR0031 PDXs. As displayed in Suppl. Figure 6C, DS-8201a induced a CR in this PDX, refractory to trastuzumab, dual-HER2 blockade and T-DM1.

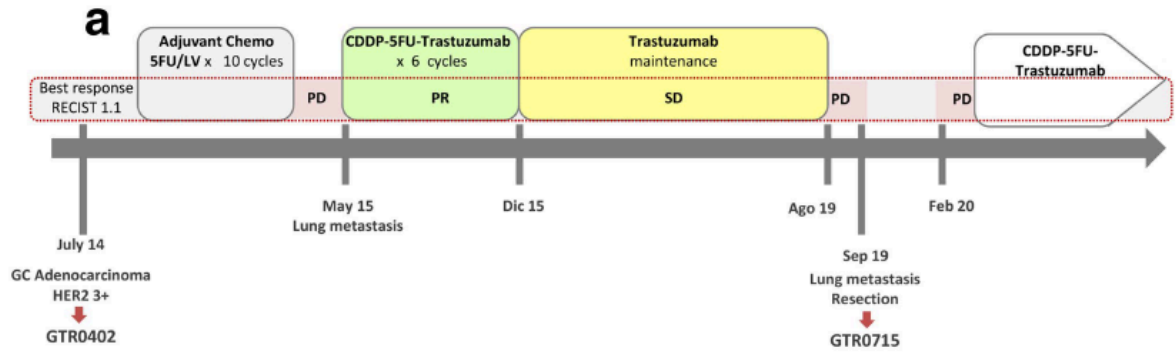
Intriguingly, we noticed that the two PDX models presenting 8–10 *HER2* copies (namely GTR0109 and GTR0031) did not show response to trastuzumab and did not get any benefit from the combo treatment. We thus performed an additional trial on another available model, GTR0471, displaying the same range of *HER2* copies. As for the other two cases presenting a similar level of *HER2* amplification, we did not observe response to neither trastuzumab nor combos (Suppl. Figure 7A), further suggesting that *HER2* may not be a dominant driver when showing this level of amplification.

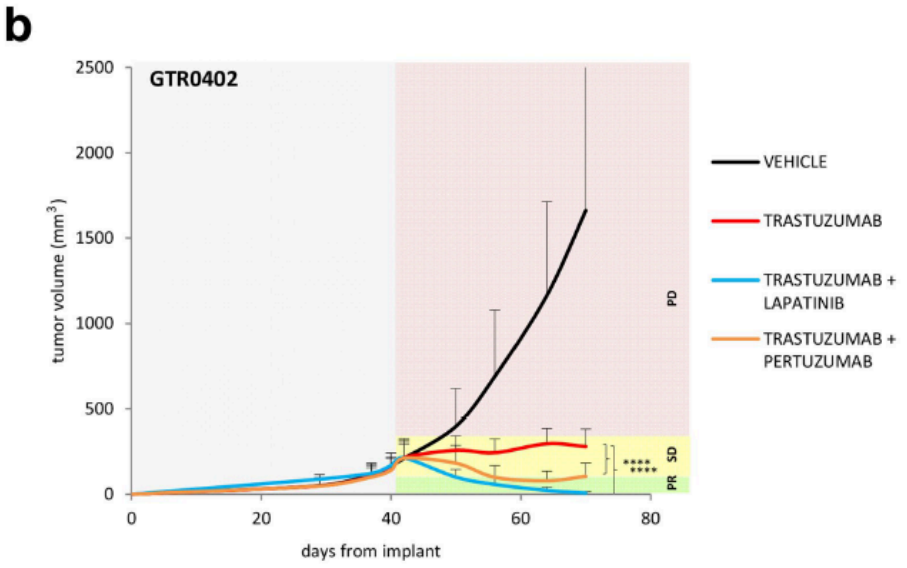
In line with this idea, we tested if *HER2*-positive GCs with 3 to 6 gene copies are resistant to these treatments as well. Since in vitro experiments performed on PDX-derived cells with high *HER2* copies were highly concordant with the in vivo results, we performed experiments on PDX-derived cells (GTR0566 and GTR0734) displaying *HER2* amplification at low copies (3–5 and 4–6, respectively). The same experiments were also conducted on established cell lines with different levels of *HER2* amplification (Suppl. Figure 7B). While all the hyperamplified cells (both primary and established) showed a response to trastuzumab, further improved by the combo, low amplified cells did not respond neither to trastuzumab nor to the combo. Even if larger cohort of cases are needed, the presented data further reinforce the idea that a level of *HER2* amplification higher than 8–10 copies is required for HER2 to be categorized as a driver of oncogene addiction.

PDX 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 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 (Fig. 4a depicts the clinical history of this patient). In the GTR0402 PDX model derived from the primary gastric adenocarcinoma (68 *HER2* copies, Suppl. Table 1), we observed SD in response to trastuzumab (Fig. 4b upper graph), 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 lapatib 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 Fig. 4b, 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 (Fig. 4a), we could derive another PDX model (GTR0402_METS; 80 HER2 copies, Suppl. Table 1) that was expanded and randomized in the same cohorts as the PDX derived from the primary tumor (Fig. 4b, lower graph). 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


a
Adjuvant Chemo
5FU/LV x 10 cycles
CDDP-5FU-Trastuzumab
x 6 cycles
Trastuzumab
maintenance
CDDP-5FU-
Trastuzumab
Best response
RECIST 1.1
PD
PR
SD
PD
PD
May 15
Lung metastasis
Dic 15
Ago 19
Feb 20
July 14
GC Adenocarcinoma
HER2 3+
GTR0402
Sep 19
Lung metastasis
Resection
GTR0715


b
GTR0402
tumor volume (mm3)
days from implant
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TRASTUZUMAB
TRASTUZUMAB +
LAPATINIB
TRASTUZUMAB +
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PD
SD
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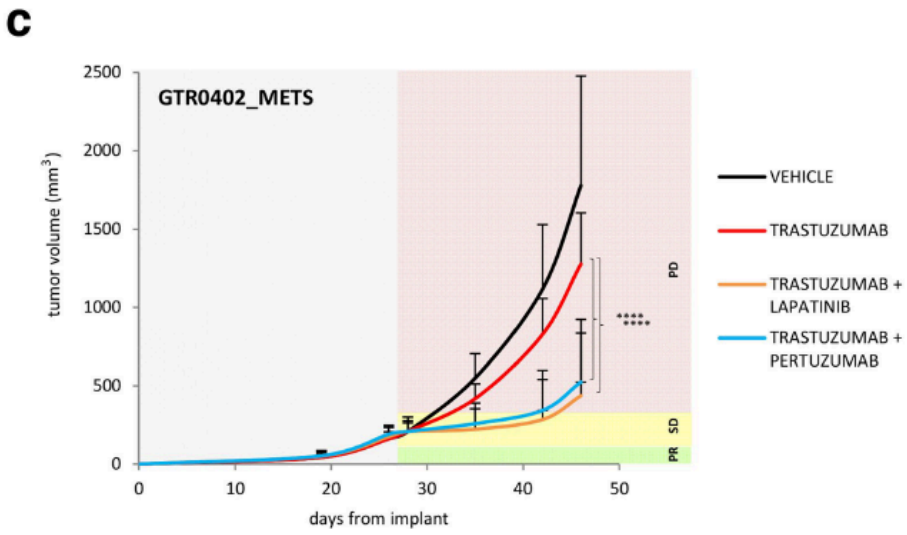
c
GTR0402_METS
tumor volume (mm3)
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VEHICLE
TRASTUZUMAB
TRASTUZUMAB +
LAPATINIB
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PERTUZUMAB
PD
SD
PR
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◂**Fig. 4** PDX models recapitulate patients' response to trastuzumab. **a** Summarized clinical course of the GTR0402 patient. Grey-lined boxes indicate periods of administration of the indicated therapeutic agents. Grey vertical lines indicate timing of tumor specimen acquisition from surgical procedures or biopsies, as well as dates of tumor assessment by CT scan. *PD* progressive disease, *PR* partial response, *SD* stable disease (according to RECIST 1.1). The two red arrows indicate timing of specimen acquisition from which PDXs were derived. *5FU/LV* 5-fluorouracil/leucovorin, *CDDP-5FU* cisplatin, 5-fluorouracil. **b** Tumor growth curves in mice cohorts derived from the GTR0402 tumor (upper graph) or from the GTR0402 metastasis (GTR0402_METS, lower graph), treated with vehicle, trastuzumab or the combos. 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

trastuzumab alone, inducing a temporary stabilization of disease. A much stronger response was induced by DS-8201a which led to tumor regression (Suppl. Figure 6D), proving the activity of this drug conjugate also in the context of acquired resistance.

PDX0455 (80 *HER2* gene copies, Suppl. Table 1) was derived from a biopsy of a tumor showing primary resistance to trastuzumab-containing treatment (progressive disease according to RECIST 1.1 criteria; Fig. 5a). Genomic analysis of the primary tumor and of the derived GTR0455 PDX model revealed the presence of an activating *HER2* mutation (p.S310Y [32]) at the allelic frequency of 95% (Fig. 5b). 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 to the clinical history of the donor patient, trastuzumab-treated GTR0455 mice were resistant to treatment and experienced disease progression (Fig. 5c). No response was observed in lapatinib-treated mice but the combination trastuzumab plus lapatinib resulted in a strong reduction of tumor volume (Fig. 5c, Suppl. Figure 8). In vitro experiments performed in PDX-derived cells (which maintained *HER2* amplification and mutation, Suppl. Figure 8B and data not shown) exhibited poor susceptibility to either trastuzumab or lapatinib used as single-agents, but strong inhibition when used in combination (Suppl. Figure 8C, D).

Overall our results show that the PDX models, in spite of the tumor heterogeneity, closely mirror the patient behaviour and thus represent an invaluable tool to test new therapeutic approaches.

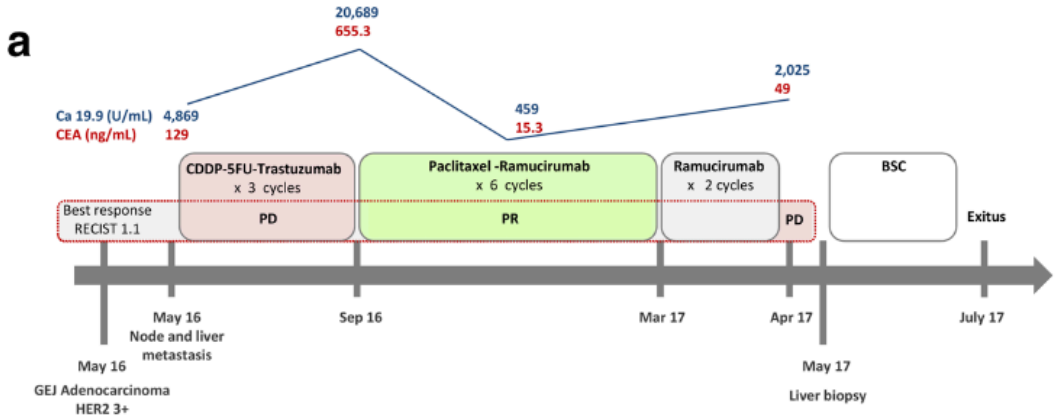
A non-cell autonomous mechanism sustains adaptive secondary resistance to HER2 inhibition

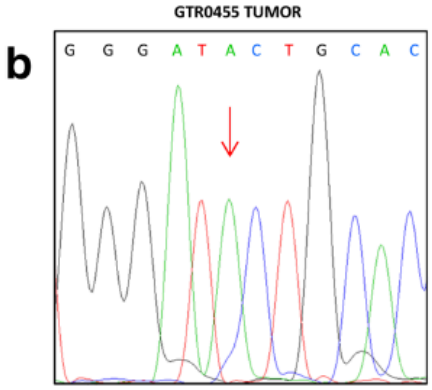
As already shown (Fig. 2), prolonged treatment of the GTR0233 PDX with anti-HER2 compounds in monotherapy resulted in tumor relapse (Figs. 2b, 6a). The genomic analysis of resistant tumors did not show any putative genomic alterations likely sustaining resistance to HER2 inhibition (data not shown). We thus investigated the onset of "adaptive" resistance sustained by activation of other receptor tyrosine kinases which could vicariate for HER2 activation. We have recently shown that TKIs can induce non-cell-autonomous adaptive resistance to MET and EGFR targeted therapies through the secretion by cancer-associated fibroblasts of the MET ligand, hepatocyte growth factor (HGF) [33]. We thus wondered if this could be true also for HER2. Immunohistochemistry analyses showed increased phosphorylation of the MET receptor in lapatinib-resistant tumors compared to the matching sensitive ones (Fig. 6b). In situ hybridization with a mouse HGF RNA probe revealed that stroma of resistant tumors produced significantly more HGF than sensitive ones (Fig. 6c, d). Then, we isolated and grew in culture Cancer-Associated Fibroblasts (CAFs), both from wild type (sensitive) and resistant tumors. PCR analysis performed on CAF mRNA (Fig. 6e) and ELISA assay (Fig. 6f) conducted on culture supernatants showed that CAFs obtained from resistant tumors produced higher amount of HGF compared to wt 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 Fig. 6g, 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.

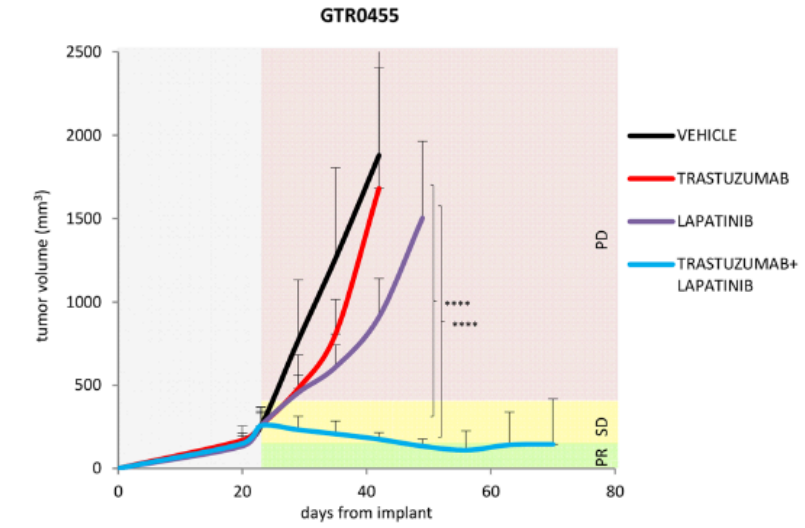
Discussion

Based on the results of the ToGA trial [3], the combination of chemotherapy with trastuzumab is considered the gold standard of treatment for patients with HER2-positive metastatic gastric cancer. However, less than 20% of patients clearly benefit from this treatment. In breast cancer, the double HER2 block provided by combining trastuzumab with pertuzumab has shown significantly better efficacy than trastuzumab monotherapy [34]. The same strategy was assessed in HER2-positive advanced gastric cancer patients by the JACOB trial which compared first-line chemotherapy plus trastuzumab and pertuzumab with standard "ToGA" strategy. Even if median OS was non-significantly increased in the experimental arm, the formally negative results of the study reinforced the well-known questions about the real role of HER2 as a unique and dominant driver in GC [10]. From this perspective,





Genealogy	Canonical Variant Classification	Gene	Canonical Protein Change	Canonical cDNA Change	dbsnp site	Allele fraction
GTR0455PDX	Missense	*HER2*	p.S310Y	c.929C>A	COSMIC	0,952



◂**Fig. 5** The trastuzumab/lapatinib combo overcomes resistance to trastuzumab monotherapy in a HER2-mutated primary resistant PDX. **a** Summarized clinical course of the GTR0455 patient. Grey-lined boxes indicate periods of administration of the indicated therapeutic agents. Grey vertical lines indicate timing of tumor specimen acquisition from surgical procedures or biopsies, as well as dates of tumor assessment by CT scan. *PD* progressive disease, *PR* partial response according to RECIST 1.1. The red arrow indicates timing of specimen acquisition from which PDX was derived. *CDDP-5FU* cisplatin, 5-fluorouracil, *BSC* best supportive care. **b** Detection of the *HER2 S310Y* mutation in the original tumor (Sanger sequencing, left panel) and in the GTR0455 PDX (exome sequencing, right panel). **c** 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

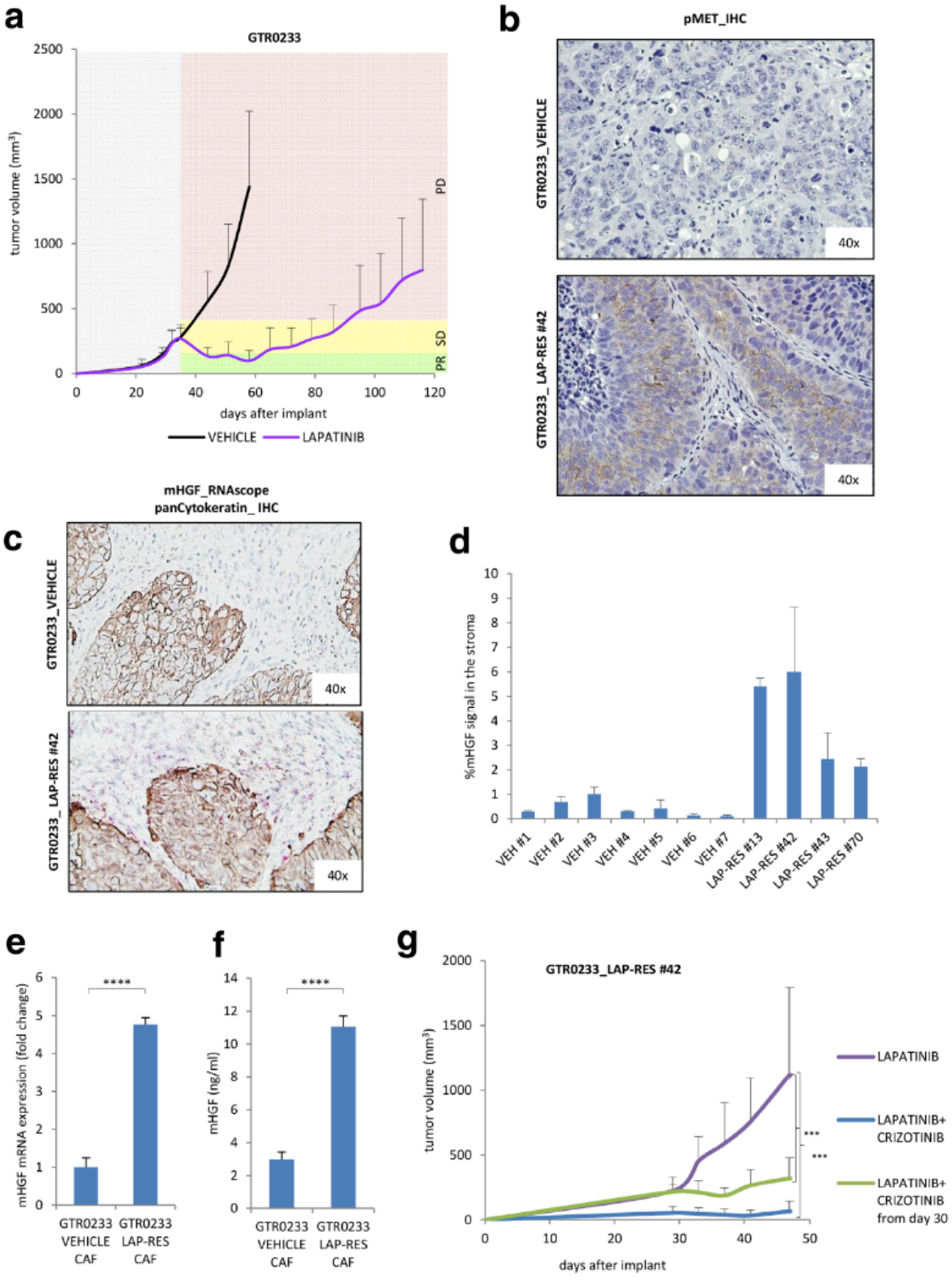
Gomez-Martin and colleagues showed that higher level of *HER2* amplification significantly predicts increased benefit from trastuzumab-based therapy in patients with advanced GC [16]. A mean *HER2/CEP17* ratio of 4.7 was found as the optimal cutoff value identifying tumors where *HER2* acts as a driver gene. Taking advantage of the unique opportunity provided by our wide platform of *HER2* "hyper"-amplified GC PDXs, we compared the efficacy of trastuzumab monotherapy versus dual therapy (tratuzumab + pertuzumab or lapatinib) in this subpopulation of HER2-positive cancers theoretically responsive to trastuzumab. Our results show that despite the high level of *HER2* amplification, 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 WT and promotes oncogenic signaling in a HER2-dependent manner [25]. 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.

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 + mGC or gastroesophageal junction [10]. However, no post hoc molecular analyses have been performed up to date to identify the molecular profile of patients who may benefit 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, indeed, we observed more that 90% of positivity of tumor cells only in hyperamplified 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. Moreover, in our small cohort of xenopatients, we noticed that the three cases harbouring 8–10 *HER2* gene copy number 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 strenghten 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 frequence 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, Kadcycla). This agent showed efficacy in one of the two models (GTR0109).


a
GTR0233
tumor volume (mm³)
2500
2000
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20
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days after implant
PD
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PR
VEHICLE
LAPATINIB
b
pMET_IHC
GTR0233_VEHICLE
40x
GTR0233_ LAP-RES #42
40x
c
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panCytokeratin_ IHC
GTR0233_VEHICLE
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d
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VEH #2
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VEH #4
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VEH #7
LAP-RES #13
LAP-RES #42
LAP-RES #43
LAP-RES #70
e
mHGF mRNA expression (fold change)
6
5
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GTR0233 VEHICLE CAF
GTR0233 LAP-RES CAF
f
mHGF (ng/ml)
14
12
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GTR0233 VEHICLE CAF
GTR0233 LAP-RES CAF
g
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tumor volume (mm³)
2000
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500
0
0
10
20
30
40
50
days after implant
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LAPATINIB
LAPATINIB+ CRIZOTINIB
LAPATINIB+ CRIZOTINIB from day 30

◂**Fig. 6** Identification of a non-cell autonomous, HGF-dependent mechanism of resistance to HER2 inhibition. **a** Generation of a lapatinib-resistant tumor. PDX GTR0233 has undergone 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, as in Fig. 2. **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 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 (CAF) 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 CAFsderived 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

Interestingly, in the resistant model (GTR0031), the new and more potent ADC trastuzumab deruxtecan (DS-8201a) was highly active. Intriguingly, the latter PDX model is *KRAS* co-amplificated (8 copies), which is a well-known biomarker of primary resistance to therapies targeting upstream receptors [8]. Indeed, the co-occurrence of *HER2* amplification and *KRAS* genomic alterations has been observed in 5% of GC patients in the TCGA database. From our experience, we have observed it in 10% of patients included in the AMNESIA study (4 out of 37 patients, all resistant to trastuzumab [8]) and in 10% of patients in our cohort (considering ≥ 4 *HER2* and *KRAS* gene copies; 3% considering ≥ 8 *HER2* and *KRAS* gene copies; data not shown). While the highly promising activity of trastuzumab deruxtecan has been recently reported in a small cohort of patients with trastuzumab-resistant *HER2*-amplified GC [31], 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.

Taken together, our results suggest 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.

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Author contributions SG and SC conceived and supervised the study and wrote the manuscript; SU, C.Migliore and FP contributed to design the experiments; MD, RR, UF, SDP, CB, GS, ER, GLB, S.Molfino, MB, MS, CC, SS, ASB, FT, FM, AR, MP, DR provided patient material and data; C.Migliore, AP, LDE, SD, DMR, SEB, SU, SR, MA, TC, S. Ribisi performed experiments; SEB performed bioinformatics analyses; IS, PC, IR, AG, A.Sapino, C.Marchio' performed the pathologic analysis; S.Guarrera performed epigenomic analyses; A.Sottile provided technical support; S.Marsoni managed the GEA Study. All the authors revised the manuscript.

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Compliance with ethical standards

Conflict of interest FP received honoraria from Amgen, Roche, Lilly, Bayer, Servier, Merck-Serono, Sanofi. Research grants from BMS; AS-B. participated in advisory boards for Amgen, Bayer, Samsung Bioepis and Sanofi. S. S is advisory board member for Amgen, Bayer, BMS, CheckmAb, Clovis, Daiichi-Sankyo, Merck, Roche-Genentech, and Seattle Genetics. CM has received personal/consultancy fees from Axiom Healthcare Strategies, Daiichi-Sankyo, MSD, Roche and Bayer.The remaining authors declare no potential conflicts of interest.

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Authors and Affiliations

Stefano Ughetto[1,2] · Cristina Migliore[1,2] · Filippo Pietrantonio[3,4] · Maria Apicella[2] · Annalisa Petrelli[2] · Laura D'Errico[1,2] · Stefania Durando[2] · Daniel Moya-Rull[2] · Sara E. Bellomo[1,2] · Sabrina Rizzolio[2] · Tania Capelôa[2,16] · Salvatore Ribisi[2] · Maurizio Degiuli[5] · Rossella Reddavid[5] · Ida Rapa[5] · Uberto Fumagalli[6,17] · Stefano De Pascale[6,17] · Dario Ribero[2] · Carla Baronchelli[7] · Giovanni Sgroi[8] · Emanuele Rausa[8] · Gian Luca Baiocchi[9] · Sarah Molfino[9] · Stefania Manenti[7] · Maria Bencivenga[10] · Michele Sacco[10] · Claudia Castelli[18] · Salvatore Siena[4,11] · Andrea Sartore-Bianchi[4,11] · Federica Tosi[4,11] · Federica Morano[3] · Alessandra Raimondi[3] · Michele Prisciandaro[3,4] · Annunziata Gloghini[12] · Silvia Marsoni[13] · Antonino Sottile[2] · Ivana Sarotto[2] · Anna Sapino[2,14] · Caterina Marchiò[2,14] · Paola Cassoni[14] · Simonetta Guarrera[2,15] · Simona Corso[1,2] · Silvia Giordano[1,2]

1 Department of Oncology, University of Torino, Candiolo, Italy

2 Candiolo Cancer Institute, FPO-IRCCS, Strada Provinciale 142, Candiolo, 10060 Turin, Italy

3 Medical Oncology Department, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

4 Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy

5 Department of Oncology, University of Torino, Orbassano, Italy

6 Chirurgia Generale 2, Spedali Civili, Brescia, Italy

7 Department of Pathology, ASST Spedali Civili, Brescia, Italy

8 Surgical Oncology Unit, Surgical Science Department, ASST Bergamo Ovest, Treviglio, BG, Italy

9 Department of Clinical and Experimental Sciences, Surgical Clinic, University of Brescia, Brescia, Italy

10 Section of Surgery, Department of Surgical Sciences, Dentistry, Gynecology and Pediatrics, University of Verona, Verona, Italy

11 Niguarda Cancer Center, Grande Ospedale Metropolitano Niguarda, Milan, Italy

12 Pathology and Laboratory Medicine Department, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

13 Istituto FIRC di Oncologia Molecolare (IFOM), Milan, Italy

14 Department of Medical Sciences, University of Torino, Turin, Italy

15 Italian Institute for Genomic Medicine, IIGM, Candiolo, Italy

16 Present Address: Pole of Pharmacology and Therapeutics, Institut de Recherche Expérimentale et Clinique (IREC),

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Optimized EGFR Blockade Strategies in *EGFR* Addicted Gastroesophageal Adenocarcinomas

Simona Corso[1,2], Filippo Pietrantonio[3,4], Maria Apicella[2], Cristina Migliore[1,2], Daniela Conticelli[1,2], Annalisa Petrelli[2], Laura D'Errico[1,2], Stefania Durando[2], Daniel Moya-Rull[2], Sara E. Bellomo[2], Stefano Ughetto[1,2], Maurizio Degiuli[5], Rossella Reddavid[5], Uberto Fumagalli[6], Stefano De Pascale[6], Giovanni Sgroi[7], Emanuele Rausa[7], Gian Luca Baiocchi[8], Sarah Molfino[8], Giovanni De Manzoni[9], Maria Bencivenga[9], Salvatore Siena[4,10], Andrea Sartore-Bianchi[4,10], Federica Morano[3], Salvatore Corallo[3], Michele Prisciandaro[3], Maria Di Bartolomeo[3], Annunziata Gloghini[11], Silvia Marsoni[2], Antonino Sottile[2], Anna Sapino[2,12], Caterina Marchiò[2,12], Asa Dahle-Smith[13], Zosia Miedzybrodzka[14], Jessica Lee[15], Siraj M. Ali[15], Jeffrey S. Ross[15,16], Brian M. Alexander[15], Vincent A. Miller[15], Russell Petty[17], Alexa B. Schrock[15], and Silvia Giordano[1,2]

ABSTRACT

Purpose: Gastric and gastroesophageal adenocarcinomas represent the third leading cause of cancer mortality worldwide. Despite significant therapeutic improvement, the outcome of patients with advanced gastroesophageal adenocarcinoma is poor. Randomized clinical trials failed to show a significant survival benefit in molecularly unselected patients with advanced gastroesophageal adenocarcinoma treated with anti-EGFR agents.

Experimental Design: We performed analyses on four cohorts: IRCC (570 patients), Foundation Medicine, Inc. (9,397 patients), COG (214 patients), and the Fondazione IRCCS Istituto Nazionale dei Tumori (206 patients). Preclinical trials were conducted in patient-derived xenografts (PDX).

Results: The analysis of different gastroesophageal adenocarcinoma patient cohorts suggests that *EGFR* amplification drives aggressive behavior and poor prognosis. We also observed that EGFR inhibitors are active in patients with *EGFR* copy-number gain and that coamplification of other receptor tyrosine kinases or *KRAS* is associated with worse response. Preclinical trials performed on *EGFR*-amplified gastroesophageal adenocarcinoma PDX models revealed that the combination of an EGFR mAb and an EGFR tyrosine kinase inhibitor (TKI) was more effective than each monotherapy and resulted in a deeper and durable response. In a highly *EGFR*-amplified nonresponding PDX, where resistance to EGFR drugs was due to inactivation of the *TSC2* tumor suppressor, cotreatment with the mTOR inhibitor everolimus restored sensitivity to EGFR inhibition.

Conclusions: This study underscores EGFR as a potential therapeutic target in gastric cancer and identifies the combination of an EGFR TKI and a mAb as an effective therapeutic approach. Finally, it recognizes mTOR pathway activation as a novel mechanism of primary resistance that can be overcome by the combination of EGFR and mTOR inhibitors.

See related commentary by Openshaw et al., p. 2964

Introduction

Gastric and gastroesophageal adenocarcinomas represent the third leading cause of cancer-related deaths worldwide. Despite the introduction of novel systemic treatment options, the outcome of patients with metastatic gastroesophageal adenocarcinoma (mGEA) is still extremely unsatisfactory, with median overall survival (OS) of less than 12 months in most clinical trials (1).

While the identification of specific molecular subtypes has had profound implications for targeted strategies in other malignancies, the same progress has only been partially realized for patients with mGEA. Trastuzumab and ramucirumab (targeting HER2 and VEGFR2, respectively) are the only approved targeted agents in mGEA (2, 3), whereas the promising role of immune checkpoint inhibitors, such as pembrolizumab and nivolumab, still needs to be confirmed by randomized clinical trials (RCT) performed in properly selected patient subgroups.

[1]Department of Oncology, University of Torino, Candiolo, Torino, Italy. [2]Candiolo Cancer Institute, FPO-IRCCS, Candiolo, Torino, Italy. [3]Department of Medical Oncology, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy. [4]Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy. [5]Department of Oncology, University of Torino, Orbassano, Torino, Italy. [6]Chirurgia Generale 2, Brescia, Italy. [7]Surgical Oncology Unit, Department of Surgical Science, ASST Bergamo Ovest, Treviglio, Bergamo, Italy. [8]Department of Clinical and Experimental Sciences, Surgical Clinic, University of Brescia, Brescia, Italy. [9]Department of Surgical Sciences, Dentistry, Gynecology and Pediatrics, Section of Surgery, University of Verona, Verona, Italy. [10]Niguarda Cancer Center, Grande Ospedale Metropolitano Niguarda, Milan, Italy. [11]Department of Pathology and Laboratory Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy. [12]Department of Medical Sciences, University of Torino, Torino, Italy. [13]Tayside Cancer Centre, Ninewells Hospital, Dundee, Scotland, United Kingdom. [14]University of Aberdeen, Aberdeen, Scotland, United Kingdom. [15]Foundation Medicine, Inc., Cambridge, Massachusetts. [16]Department of Pathology, Upstate Medical University, Syracuse, New York. [17]Division of Molecular and Clinical Medicine, School of Medicine, University of Dundee, Dundee, Scotland, United Kingdom.

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S. Corso, F. Pietrantonio, M. Apicella, and C. Migliore contributed equally to this article.

Current address for U. Fumagalli, S. De Pascale, and S. Marsoni: Chirurgia dell'Apparato Digerente – European Institute of Oncology IRCCS – Milan, Italy.

Corresponding Authors: Silvia Giordano, Department of Oncology, University of Torino, Candiolo Cancer Institute - FPO, IRCCS, sp 142, Candiolo, Torino 10060, Italy. Phone: 3901-1993-3233, ext. 3238; Fax: 3901-1993-3225; E-mail: silvia.giordano@unito.it; and Simona Corso, E-mail: simona.corso@unito.it

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Translational Relevance

Prior clinical trials performed in unselected patients with gastroesophageal adenocarcinoma failed to show survival improvement upon treatment with anti-EGFR therapies. We report the clinical activity of EGFR mAbs in patients bearing a high level (>8 copies) of *EGFR* gene amplification, and show that in patient-derived xenografts, the combination of an EGFR mAb and a tyrosine kinase inhibitor (TKI) is significantly more effective and long lasting than mAb monotherapy. We also identify mTOR pathway activation as a novel mechanism of resistance to EGFR-targeted therapy and show that it can be overcome by the combination of EGFR/mTOR inhibitors. These findings recognize EGFR as an actionable target in a small but significant subgroup of patients bearing *EGFR* amplification and suggest the combination of an EGFR mAb and a TKI as the most effective treatment.

The molecular landscape of gastroesophageal adenocarcinoma has been extensively described and the two main molecular classifications (4, 5) identified a disease subtype characterized by chromosomal instability and amplification of receptor tyrosine kinases (RTK). *EGFR* amplification has been reported in 3%–5% of gastroesophageal adenocarcinomas (4, 6), while other genetic alterations (such as point mutations or translocations) are extremely uncommon. Several EGFR-targeting drugs, comprising mAbs and tyrosine kinase inhibitors (TKI), have been approved for the treatment of multiple tumor types, including *RAS* wild-type metastatic colorectal cancer, head and neck squamous cell carcinoma, and *EGFR*-mutated advanced non–small cell lung cancer (7). Conversely, three phase III RCTs evaluating the addition of cetuximab, panitumumab, or gefitinib to the standard of care in molecularly unselected patients with advanced gastric or esophageal adenocarcinomas reported negative results (8–10). On the other hand, intriguingly, experimental data obtained in gastroesophageal adenocarcinoma preclinical models showed a positive correlation between cetuximab response and high EGFR expression/amplification (11). Consistent with these preclinical findings, the association between *EGFR* copy-number gain (CNG) and better OS has been shown by a phase II trial of cetuximab plus FOLFOX chemotherapy in patients with mGEA (12). In addition, a prespecified subgroup analysis of the COG trial showed that patients with esophageal and gastroesophageal junction carcinomas bearing *EGFR* CNG derived a significant progression-free survival, OS, and health-related quality of life benefit from gefitinib compared with placebo, thereby providing the proof of concept for *EGFR* CNG as a predictive biomarker of efficacy of EGFR-targeted agents (13).

Here, we aimed to investigate the efficacy of several EGFR inhibition strategies in preclinical models of *EGFR*-amplified gastroesophageal adenocarcinomas, to describe the clinical and molecular features of patients with *EGFR*-amplified tumors and their responsiveness to EGFR inhibition, and to extensively investigate common and potentially novel genomic mechanisms of resistance, with the ultimate goal to optimize EGFR-targeted combinations for the development of future clinical trials.

Materials and Methods

Patients

IRCC

Tumor samples (from gastric and gastroesophageal junction adenocarcinomas) and matched normal samples were obtained from patients undergoing surgery in 15 Italian hospitals: Candiolo Cancer institute-FPO, IRCCS (Torino, Italy), Ordine Mauriziano Hospital (Torino, Italy), San Giovanni Battista Hospital (Torino, Italy), San Luigi Gonzaga Hospital (Torino, Italy), Humanitas-IRCCS (Milano, Italy), San Raffaele Hospital (Milano, Italy), Treviglio-Caravaggio Hospital (Bergamo, Italy), Brescia Hospital (Brescia, Italy), Borgo-Trento Hospital (Verona, Italy), Santa Maria delle Scotte Hospital (Siena, Italy), Forlì Hospital (Forlì, Italy), Fondazione Macchi Hospital (Varese, Italy), Pisa Hospital (Pisa, Italy), Fondazione IRCCS Istituto Nazionale dei Tumori (Milano, Italy), and Ospedale Niguarda Ca' Granda (Milano, Italy). 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 performed in accordance with the principles of the Declaration of Helsinki, the International Conference on Harmonisation and Good Clinical Practice guidelines, and General Data Protection Regulation. Clinical and pathologic data were entered and maintained in our prospective database. All the samples were anonymized before being shipped to Candiolo. No reference to the patients can be inferred from the histologic and molecular characterization presented in the work.

Foundation Medicine, Inc.

Tumor samples from patients with gastroesophageal adenocarcinoma were submitted during routine clinical care for comprehensive genomic profiling (CGP). Approval for this study, including a waiver of informed consent and a Health Insurance Portability and Accountability Act waiver of authorization, was obtained from the Western Institutional Review Board (protocol no. 20152817).

Cell lines and drugs

293T cells were obtained from the ATCC and OE21 from Sigma-Aldrich. The genetic identity of the cell lines was confirmed by short tandem repeat profiling (Cell ID, Promega). Erlotinib and everolimus were purchased from Carbosynth. Cetuximab and lapatinib were provided by the hospital pharmacy.

Primary cell cultures and organoids

Gastroesophageal adenocarcinoma primary cells were derived from patient-derived xenografts (PDX) as described in (14), while gastroesophageal adenocarcinoma primary organoids were obtained as described in (15). The genetic identity of the *in vitro*–derived material with the original tumor was verified by short tandem repeat profiling (Cell ID, Promega). GTR0078 cells were used for the *in vitro* experiments soon after tumor dissociation, as they do not permanently grow in culture.

Western blot analysis and immunoprecipitation

Cells/organoids were treated with the indicated drugs: 100 nmol/L lapatinib or erlotinib for 2 hours and 0.5 μg/mL cetuximab for 16 hours. Whole-protein extracts were prepared using Laemmli buffer and quantified using the BCA Protein Assay Kit (Pierce). EGFR immunoprecipitation was performed with cetuximab on organoids (stimulated with 100 ng/mL EGF for 15 minutes, treated or not with erlotinib 100 nmol/L for 2 hours) previously washed out from Matrigel with Cell Recovery Solution (#354253, Corning) and lysed with EB (1% Triton, 20 mmol/L Tris-HCl pH 7.4, 5 mmol/L EDTA pH 8, 10% glycerol, and 150 mmol/L NaCl). Primary antibodies, anti-EGFR (1005: sc-03) and anti-Actin, were from Santa Cruz Biotechnology, and antibodies against phosphorylated EGFR (Tyr 845), ERK (Thr202/Tyr204), phosphorylated AKT (Ser473) (Clone D9E), total AKT, and ERK were from Cell Signaling Technology. Antibody against phosphorylated

EGFR (Tyr1068) (ab5644) was from Abcam. Antibody directed against amino acid 1,172–1,186 of human EGFR was described in (16). Antibody anti-EGFR extracellular epitope (111.6 antibody) was from Thermo Fisher Scientific. Secondary antibodies were from Amersham. Detection was performed with ECL System (Amersham).

Transfection and transduction procedures

OE21 cells were transfected with siRNAs using Lipofectamine 2000 (Thermo Fisher Scientific). Transfection reagents plus siRNAs at final concentration of 20 nmol/L were used following standard protocols. Seventy-two hours after transfection, cells were lysed and Western blotting was performed. *TSC2* silencing was achieved by using SMARTpool ON-TARGETplus siRNA (Dharmacon).

Lentiviruses were produced as described in (17). OE21 cells were transduced with a pool of lentiviral particles containing four *TSC2* silencing short hairpin RNAs (shRNA; Sigma, #40179, #40178, #40454, and #40455). Cells were selected with puromycin, checked for *TSC2* silencing, and subcutaneously injected in NOD/SCID mice (5×10^6 cells/mouse) in 1:1 SF medium:Matrigel (Corning).

Analyte extraction

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

Copy-number variation evaluation by qRT-PCR

Quantitative PCR experiments for estimation of *EGFR*, *MET*, *FGFR2*, and *KRAS* copy-number variations (CNV) were performed in triplicates using 2 ng total gDNA as a template, with the following human TaqMan copy-number assays: for *HER2* assay, ID Hs02876245_cn; for *EGFR* assay, ID Hs04942325_cn; for *MET* assay, ID Hs04993403_cn,; for *FGFR2* assay, ID Hs01472955_cn; for *KRAS* assay, ID Hs06936191; and the TaqMan Copy-number Reference Assay RNase P 4316831 and GREB1 Hs01738470_cn (Applied Biosystems). PCR runs were performed with ABI Prism 7900HT (Applied Biosystems).

AMNESIA panel

In a case–control study setting, we identified a panel of gene alterations (including *EGFR/MET/KRAS/PIK3CA/PTEN* mutations and *EGFR/MET/KRAS* amplifications) able to predict primary resistance to trastuzumab therapy in patients with HER2-positive metastatic gastric cancer (18). We applied the same panel of gene alterations (substituting *EGFR* mutation/amplification with *HER2* mutation/amplification) in the context of EGFR-driven tumors.

Phospho-Kinase array

Cells were treated with the indicated drugs: 100 nmol/L lapatinib or erlotinib for 2 hours and 0.5 μg/mL cetuximab for 16 hours. The analysis of the phosphorylation profiles of kinases was performed using the Human Phospho-Kinase Antibody Array (R&D Systems), according to the manufacturer's instructions. Signal quantification was performed using Image Lab 5.2.1 Software (Bio-Rad).

PDX generation

Gastric PDX generation was performed as described in (19). All animal procedures adhered to the "Animal Research: Reporting of *In Vivo* Experiments" standards and were approved by the Ethical Commission of the Candiolo Cancer Institute (Torino, Italy) and by the Italian Ministry of Health.

PDX xenotrials

PDXs were passaged and expanded for >2 generations until production of a cohort of mice. 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) orally; cetuximab 20 mg/kg, i.p., twice weekly; lapatinib 100 mg/kg, daily, orally; erlotinib 50 mg/kg, daily, orally; and everolimus 6 mg/kg, daily, orally. Tumor size was evaluated once weekly by caliper measurements and approximate volume of the mass was calculated by using the formula $4/3\pi(D/2)(d/2)^2$, where d is the minor tumor axis and D is the major tumor axis. The response in mice was evaluated using RECIST 1.1–like criteria, that is, progressive disease (PD): ≥35% increase from baseline, partial response (PR): ≥50% reduction from baseline; and stable disease (SD): intermediate variations from baseline (20). Statistical testing for pharmacologic experiment was performed with GraphPad Prism software 8.0, using two-way ANOVA, followed by Bonferroni multiple comparisons experiments. Statistical significance: ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Genomic sequencing

IRCC samples

DNA extracted from PDX models along with a sample of normal germline DNA from each patient were utilized 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 upon their alteration in prior studies of gastroesophageal cancer (21, 22).

Foundation Medicine, Inc. samples

CGP was performed in a Clinical Laboratory Improvement Amendments–certified, New York State and College of American Pathologists–accredited laboratory [Foundation Medicine, Inc. (FMI)]. In brief, ≥50 ng DNA was extracted from 40 μm of formalin-fixed, paraffin-embedded (FFPE) tissue blocks from 4,337 cases of gastric carcinoma. The samples were assayed by CGP using adaptor ligation and hybrid capture was performed for all coding exons of cancer-related genes from 180 to 395 plus select introns from 14 to 34 genes frequently rearranged in cancer. Sequencing of captured libraries was performed to a mean exon coverage depth of >500×, and resultant sequences were analyzed for genomic alterations, including mutations (base substitutions, insertions, and deletions), copy-number alterations (focal amplifications and homozygous deletions), and select gene fusions or rearrangements, as described previously (23). *EGFR* amplification was defined as *EGFR* copy ≥8.

COG samples

RTK copy numbers were determined using Affymetrix OncoScan CNV FFPE assay following the manufacturer's recommended protocol. DNA was extracted from histologically confirmed esophageal and gastroesophageal junction adenocarcinomas as described previously (13) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies) following the manufacturer's recommended protocol, using 80 ng for each case, normalized to a concentration 12 ng/μL. Array fluorescence intensity data (CEL files), generated by Affymetrix GeneChip Command Console software version 4.0, were processed using OncoScan Console software version 1.1.034 to produce OSCHP files and a set of QC metrics. Features were quantile normalized and genome-wide allele-specific copy number was assessed using the Affymetrix TuScan algorithm to allow adjustment for both tumor ploidy and nonaberrant cell admixture (24). Genome-wide

CNV was assessed across all cases using Affymetrix Nexus express for OncoScan (version 3.1.). Significant CNV events across the genome were identified using a "significance testing for aberrant copy number" (STAC) approach (25).

The Fondazione IRCCS Istituto Nazionale dei Tumori samples

FFPE archival tumor tissue blocks obtained prior to any treatment were used for the purpose of this study. Next-generation sequencing was performed, as in (26), to detect gene mutations, whereas *EGFR*, *HER2*, and *MET* status was determined by silver *in situ* hybridization (SISH) analysis and *KRAS* GCN gain was assessed by PCR, as described previously in (18).

Survival analysis

OS was calculated from the date of enrollment (for the COG trial) or from the date of diagnosis of metastatic disease [for the Fondazione IRCCS Istituto Nazionale dei Tumori (INT) dataset] until the date of death or last follow-up for alive patients. The OS curves for *EGFR*-amplified versus nonamplified subgroups were calculated with the Kaplan–Meier method and compared with the log-rank test. Survival analysis for COG was undertaken using IBM SPPS statistics 22, for further details see (10, 13).

ISH and IHC

EGFR gene status was assessed by Bright-field Dual-color SISH (Ventana Medical Systems). The Colorado scoring system was adopted to classify samples into ISH strata according to the frequency of cells with each *EGFR* gene copy number and referred to the chromosome 7 centromere. *EGFR* SISH–negative cases had no or low genomic gain for *EGFR* gene copy number (disomy, low trisomy, high trisomy, and low polysomy), whereas the distinction between high polysomy and gene amplification was defined by the presence of gene clusters only in *EGFR*-amplified cases. *EGFR* FISH in the COG cohort was performed and scored as described in (13).

IHC for EGFR was performed using the CONFIRM anti-EGFR (5B7) rabbit monoclonal primary antibody (Ventana Medical Systems) that recognizes the internal domain of EGFR and the monoclonal mouse anti-human anti-EGFR (E30) antibody (Dako) that recognizes an external domain of EGFR. IHC was carried out on an Automated Immunostainer (BenchMark Ultra; Ventana Medical Systems) using the Optiview DAB Detection Kit (Ventana Medical Systems). IHC for phosphorylated EGFR was performed using anti-phosphorylated EGFR Y1173 53AS from Cell Signaling Technology.

Transcriptome profiling

RNA sequencing (RNA-seq) libraries were prepared using the Illumina TruSeq Stranded Total RNA Library Prep Gold kit and sequenced generating 75 bp paired-end reads. PDX RNA-seq data were first deconvoluted for mouse contamination with Xenome (27) software (version 1.0.1). Nonhost reads (those classified as "graft," "ambiguous," or "both") were then mapped to UCSC hg38 reference genome with HISAT2 (28) aligner with default parameters. Gene expression estimate was performed with HTSeq (29) in "intersection-nonempty" mode against GENCODE v33 annotation.

Results

Prevalence of *EGFR* amplification in patients with gastroesophageal adenocarcinoma

We evaluated *EGFR* copy number in four different cohorts: (i) a proprietary cohort (IRCC cohort) of 570 primary gastroesophageal adenocarcinomas (real-time PCR analysis), (ii) the FMI dataset of 4,337 gastric and 5,060 esophageal/gastroesophageal junction adenocarcinomas (CGP), (iii) the subgroup of 214 patients with esophageal or gastroesophageal junction adenocarcinoma enrolled in the COG trial (NCT01243398) of second-line gefitinib versus placebo (ref. 10; FISH), and (iv) the Fondazione IRCCS INT of Milan dataset of 206 patients with mGEA (ISH and SISH). In the IRCC cohort we identified 44 primary tumors (7.8%) with *EGFR* CNG (≥4 gene copies), with 10 of them (1.8% of all samples) bearing >8 gene copies (the suggested threshold of biologically meaningful amplification in the *HER2* and *MET* context; ref. 30) and eight of them (1.4% of all samples) bearing a heterogeneous *EGFR* amplification (one tumor area >8 copies and one tumor area ≤8 copies; **Fig. 1**; Supplementary Table S1). In the FMI dataset, 3.4% of gastric and 7.6% of esophageal carcinomas showed *EGFR* amplification equal or higher than eight copies, while in the COG and INT datasets, the frequencies of *EGFR* amplification were 7% and 4.9%, respectively (**Fig. 1**). In both COG and INT cohorts, no significant association between *EGFR* amplification and baseline clinicopathologic characteristics was observed (Supplementary Tables S2 and S3).

EGFR amplification drives aggressiveness and poor prognosis in gastroesophageal adenocarcinomas

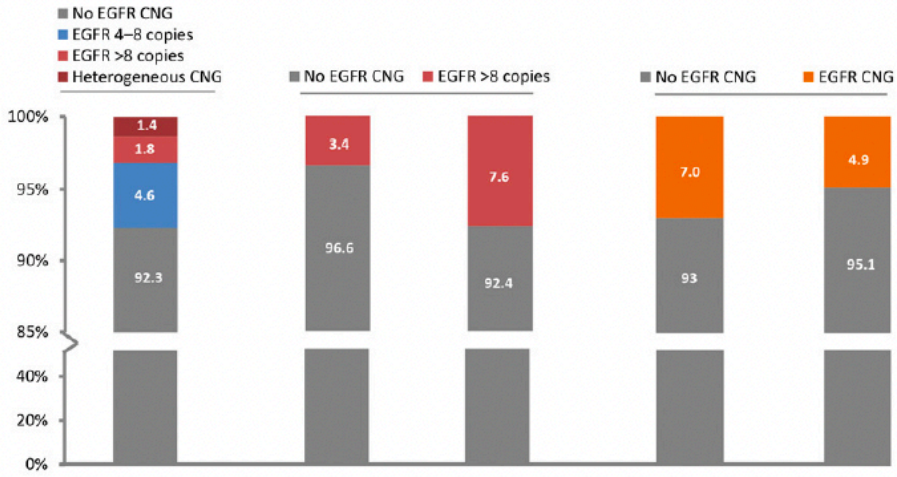
To investigate whether *EGFR* amplification is associated with poor prognosis of gastroesophageal adenocarcinomas, we took advantage of a cohort of pretreated patients with esophageal and gastroesophageal junction adenocarcinomas enrolled in the COG trial and randomized to placebo (10). Among 102 cases with available *EGFR* FISH status, patients with *EGFR* amplification had a significantly inferior median OS compared with those without *EGFR* amplification [3.1 vs. 3.5 months; HR, 1.23; 95% confidence interval (CI), 1.03–1.48; $P = 0.026$; **Fig. 2A**, left]. All patients with *EGFR*-amplified tumors died within 4 months.

Similarly, when focusing on the INT dataset, patients with *EGFR* amplification had inferior median OS as compared with those with *EGFR* SISH–negative tumors (17 vs. 18.9 months; HR, 1.95; 95% CI, 0.90–4.21; $P = 0.083$; **Fig. 2A**, right). These results have also been confirmed in primary gastric tumors by analyzing The Cancer Genome Atlas (TCGA) data, in which tumor *EGFR* amplification correlated with significantly inferior OS and disease-free survival (**Fig. 2B**).

Activity of EGFR inhibitors in patients with *EGFR*-amplified metastatic gastric cancer and landscape of primary treatment resistance

To determine whether patients with *EGFR*-amplified mGEA may respond to EGFR inhibitors and to eliminate the potentially confounding effect of the combination with chemotherapy, we focused on patients with *EGFR*-amplified mGEA treated at INT with the anti-EGFR mAb, panitumumab, as single agent after failure of standard treatment options. Three patients with *EGFR* amplification, confirmed by SISH, were identified (Supplementary Fig. S1A); their molecular profile is summarized in Supplementary Fig. S1B and their clinical history is reported in **Fig. 3**. Briefly, INT#001 patient had *KRAS*-coamplified mGEA and showed PD at the first radiological reassessment, INT#002 patient had no cooccurring alterations in *HER2*, *MET*, *KRAS*, or *PIK3CA* and showed a PR lasting 6 months, and INT#003 patient had cooccurring heterogeneous *KRAS* amplification and showed a PR lasting only 10 weeks, followed by rapid clinical progression and death.

To verify whether RTK pathway activation is associated with EGFR inhibitor resistance in gastroesophageal adenocarcinoma, we



COHORT NAME	IRCC	FMI		COG	INT
SAMPLE TYPE	Gastric/GEJ	Gastric	Esophageal/GEJ	Esophageal/GEJ	Gastric/GEJ
SAMPLE SIZE	*N* = 570	*N* = 4,337	*N* = 5,060	*N* = 214	*N* = 206
EGFR CNG DETECTION	qRT-PCR	Genomic profiling	Genomic profiling	FISH	SISH

Figure 1.

EGFR CNG. The graphs illustrate the percentage of tumors displaying *EGFR* CNG in four different cohorts. Real-time PCR analysis of IRCC gastric/gastroesophageal junction (GEJ) adenocarcinomas displaying *EGFR* gain (4–8 copies or >8 copies) or heterogeneity (significantly different *EGFR* CNG in diverse analyzed samples from the same tumor, with one tumor sample displaying >8 copies and one tumor sample having ≤8 copies). CGP of FMI gastric and esophageal/gastroesophageal junction cases, FISH analysis of COG esophageal/gastroesophageal junction cases, and SISH analysis of INT gastric/gastroesophageal junction adenocarcinomas.

investigated the relationship between RTK CNG and survival following treatment with gefitinib in 12 *EGFR* FISH–positive gastroesophageal adenocarcinomas (seven with amplification and five with high polysomy) of the COG trial. All 12 tumors analyzed had CNG (defined as ≥4 gene copies) of at least one RTK (*HER2, HER3, HER4, MET, FGFR1, FGFR2, FGFR3, FGFR4, IGF1R, PDGFR2, VEGFR1, VEGR2,* and *VEGFR3*). We found a significant inverse correlation between the extent of coamplification of the RTKs and OS (**Fig. 4A**). This observation of shorter survival following gefitinib treatment with activation of RTKs other than EGFR suggests optimizing inhibition of downstream signal transduction pathways could produce durable clinical responses.

To investigate the prevalence of potential genetic predictors of primary resistance to anti-EGFR treatment, we interrogated the TCGA dataset for the presence of resistance alterations included in our previously published AMNESIA panel (18) among cases with *EGFR* amplification and showed the cooccurrence of other genomic events in 53% of samples (Supplementary Fig. S2). Finally, because the available *in silico* datasets mainly represent a collection of primary gastroesophageal adenocarcinomas, we investigated the prevalence of AMNESIA panel alterations in the 534 samples from patients with *EGFR*-amplified mGEA included in the FMI dataset. This analysis showed the cooccurrence of other genomic events of interest in 186 (35%) samples (**Fig. 4B**).

Dual EGFR blockade is the most effective treatment for *EGFR*-amplified PDXs

Future trials might be prompted to reassess the role of anti-EGFR mAbs and TKIs, either as monotherapy or in combination, in molecularly selected patients with gastroesophageal adenocarcinoma. As already shown for dual HER2 blockade (trastuzumab plus pertuzumab or lapatinib) in HER2-positive breast and colorectal cancer (31–33), and despite the partially negative phase III data recently reported with this strategy in HER2-positive gastric cancer (34), dual EGFR blockade strategies with an anti-EGFR mAb plus a TKI may be more effective than each drug as monotherapy.

A large series of human cancer specimens transplanted into mice (PDX) produce a study population that can be randomized for prospective treatment with targeted agents and thus, provides a strong strategy to perform precision medicine preclinical studies. This approach brings together the plasticity of preclinical analysis with the informative value of population-based studies. From 570 gastric

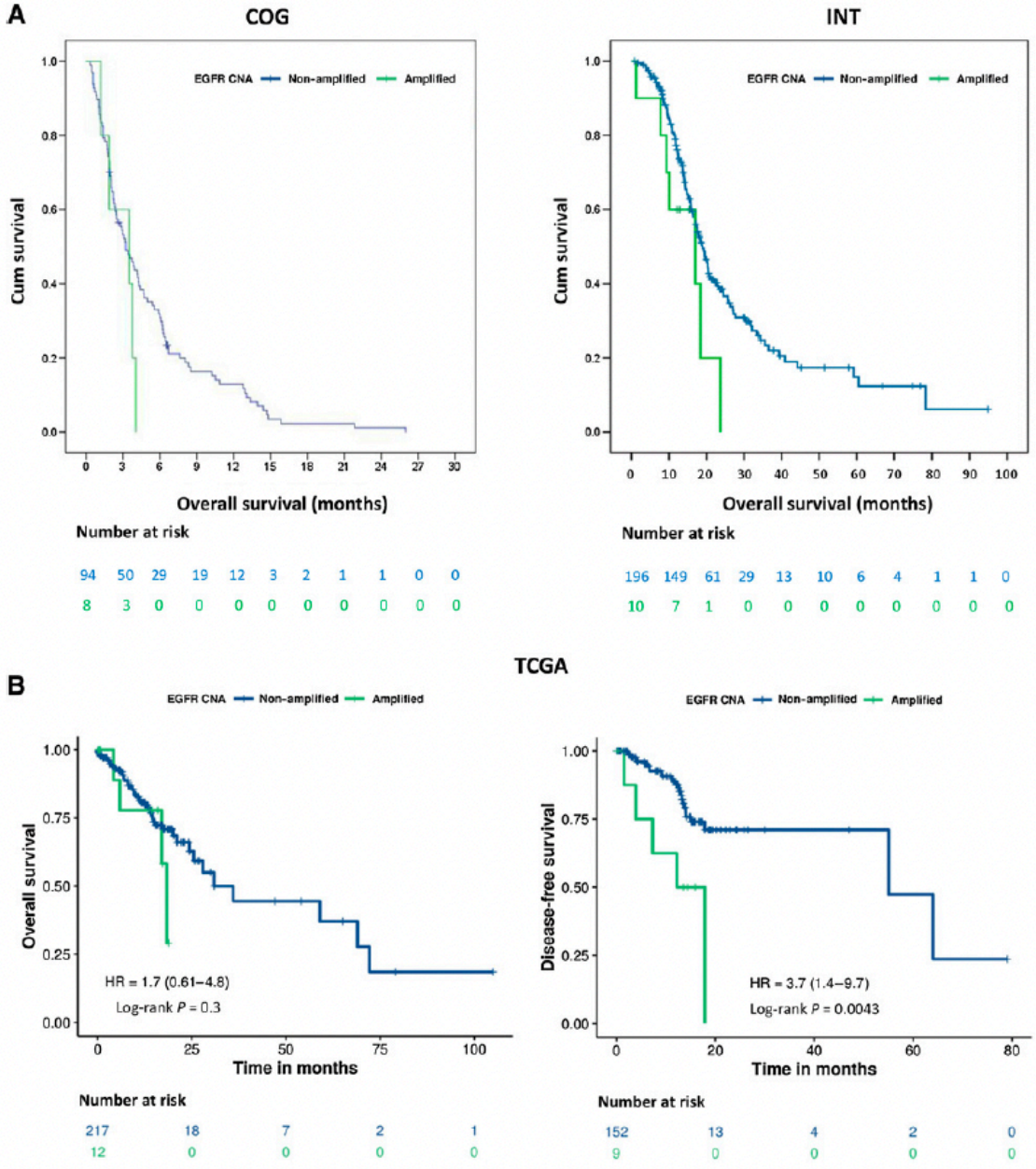


Figure 2.
Survival analysis of patients with *EGFR* CNG. **A,** The graphs show the cumulative survival (cum survival) of patients of the COG (left) and INT (right) cohorts related to *EGFR* CNG. **B,** The graphs show the OS (left) and the disease-free survival (right) of patients of the gastroesophageal TCGA dataset, related to *EGFR* CNG.

carcinoma samples (IRCC cohort), we generated a multi-level platform of gastroesophageal adenocarcinoma models, comprising 151 PDXs, primary cell lines, and organoids (22). Despite conflicting evidence on the CNG threshold clearly defining gene amplification, preclinical and clinical data obtained from gastroesophageal adenocarcinoma displaying *HER2* or *MET* amplification suggested that the clinically relevant threshold is higher than eight gene copies (30, 35). Eleven PDXs harbored at least 4–8 *EGFR* copies and four PDXs had >8 *EGFR* copies (Supplementary Fig. S3A, GTR0060: ~240 *EGFR* copies; GTR0078: ~700 copies; GTR0110: 12 copies; and GTR0511: ~80

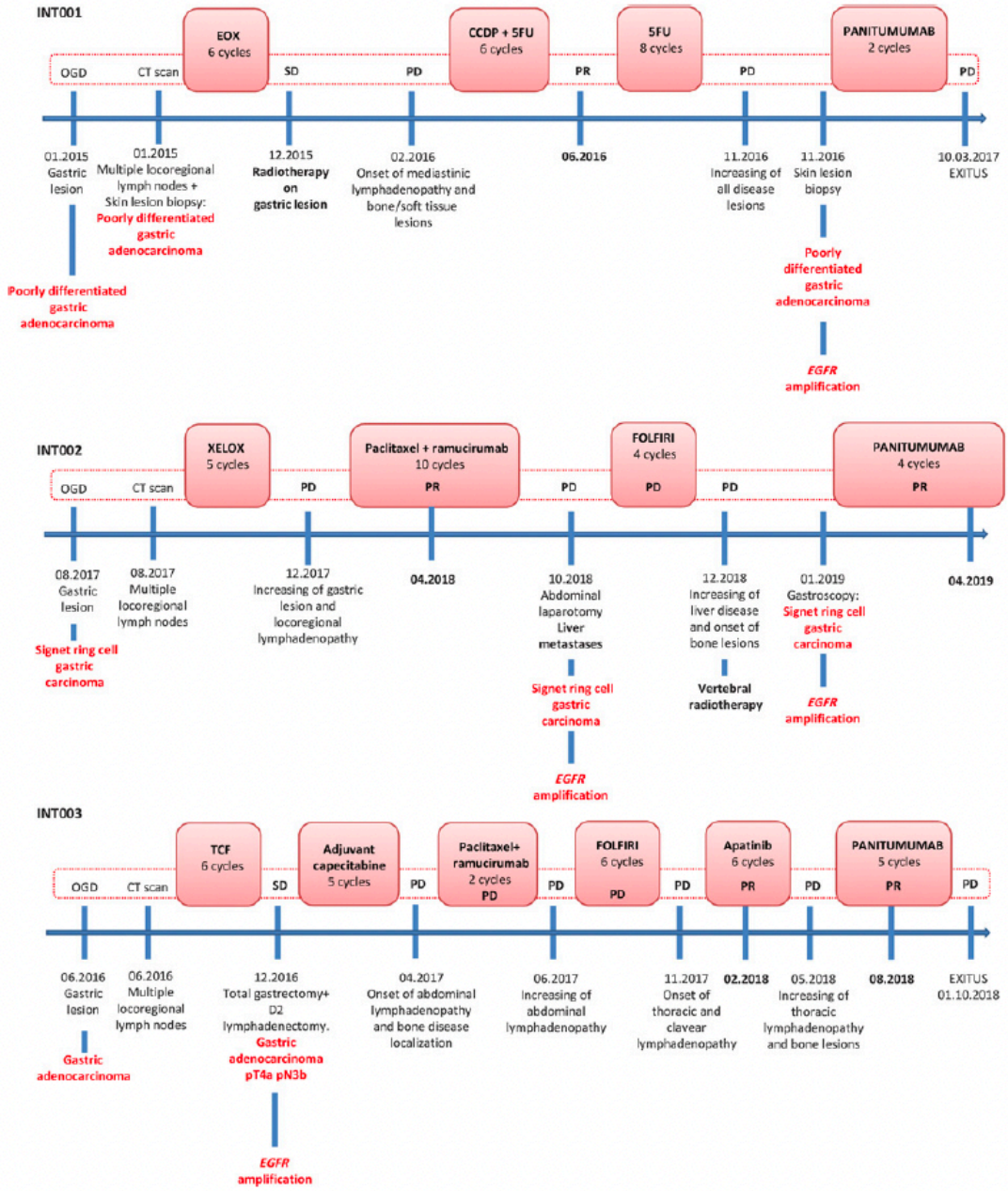
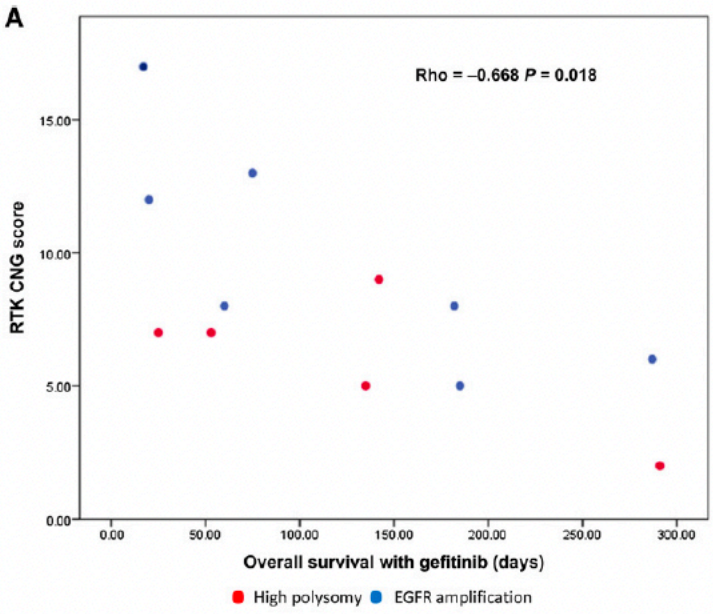


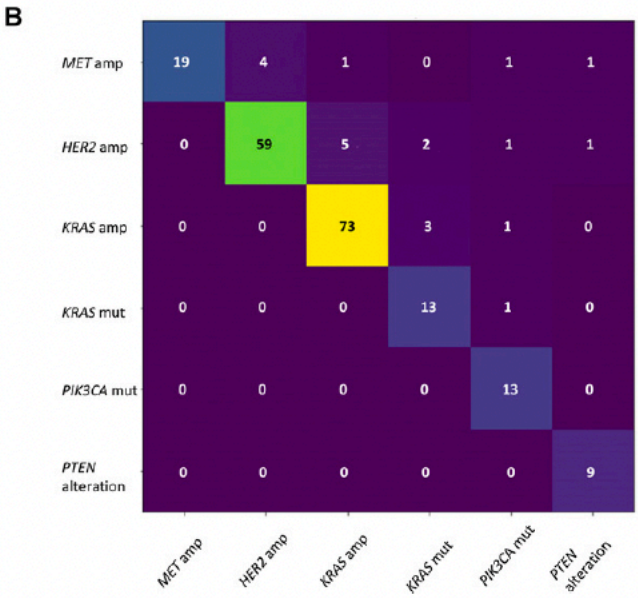
Figure 3.

Clinical history of patients treated with EGFR-targeted drugs. Summarized clinical course of INT patients with *EGFR* CNG. Red-lined boxes indicate periods of administration of the indicated therapeutic agents. Blue vertical lines indicate timing of tumor specimen acquisition from surgical procedures or biopsies, as well as dates of tumor assessment by CT scan. PD and SD according to RECIST 1.1. 5FU, 5-fluorouracil; CCDP, cisplatin, vinorelbine, ifosfamide, and epirubicin; EOX, epirubicin, oxaliplatin, and capecitabine; FOLFIRI, folinic acid, 5-fluorouracil, and irinotecan; OGD, esophago-gastro-duodenoscopy; TCF, docetaxel, carboplatin, and 5-fluorouracil; XELOX, capecitabine and oxaliplatin.

Figure 4.

RTK/KRAS pathway activation in *EGFR*-amplified cases. **A,** The scatter plot shows a significant inverse correlation between the extent of RTKs coamplification (*HER2, HER3, HER4, MET, FGFR1, FGFR2, FGFR3, FGFR4, IGF1R, PDGFR2, VEGFR1, VEGR2*, and *VEGFR3* ≥4 gene copies) and OS in 12 *EGFR* FISH–positive gastroesophageal adenocarcinomas treated with gefitinib in the COG trial. Red dots indicate cases with high polysomy and blue dots represent cases with *EGFR* amplification. **B,** The graph shows the cooccurrence of *EGFR* amplification and genomic events affecting the RTK/KRAS pathway in *EGFR*-amplified gastroesophageal adenocarcinoma tumors in the FMI dataset.





copies). These four models did not bear any other RTKs/*KRAS* CNV >8 copies (data not shown). SISH analysis and IHC confirmed uniform *EGFR* amplification and expression (Supplementary Fig. S3B). These PDX models were expanded to generate cohorts of mice to evaluate the efficacy of the EGFR mAb, cetuximab, and the TKIs, erlotinib (EGFR selective) and lapatinib (dual EGFR/HER2 inhibitor), as well as the combination of the mAb with a TKI. The original tumors were serially passaged *in vivo* until 6 tumor-bearing animals were produced per experimental group. When xenografts reached an average tumor volume of approximately 250 mm^3, mice were randomized into six independent treatment cohorts: (i) vehicle (placebo), (ii) cetuximab, (iii) erlotinib, (iv) lapatinib, (v) cetuximab + erlotinib, and (vi) cetuximab + lapatinib. Tumor response was evaluated according to RECIST-like criteria (see Materials and Methods and figure legends).

As shown in **Fig. 5A**, the GTR0060 PDX (240 *EGFR* copies) did not exhibit response to either of the TKIs used as monotherapy, but showed PR upon cetuximab treatment. Notably, both the combination (cetuximab + TKIs) treatments resulted in a complete response (CR). Interestingly, in 4 of 6 mice in the combination arms, including 3 of 3 mice treated with erlotinib + cetuximab, the tumor mass did not reappear even after more than 2 months of drug removal (**Fig. 5B**). Improved efficacy of the combination treatment was observed at long term also in a second model, GTR0110, characterized by a lower *EGFR* CNG (12 copies), uniformly distributed among tumor cells (Supplementary Fig. S3B). While neither erlotinib nor lapatinib resulted in a clinical response and cetuximab conferred disease stabilization, cetuximab plus TKI combination treatment resulted in a PR (**Fig. 5C**). Moreover, at the end of the experiment, the tumor volume was significantly reduced in mice treated with the combination compared with those treated with the mAb alone. The xenotrial performed in the GTR0511 PDX (80 *EGFR* copies) cohort also showed response to anti-EGFR treatment. Even though neither cetuximab nor lapatinib monotherapies were effective, their combination resulted in a relevant response. Interestingly, in this PDX, erlotinib was the only effective monotherapy (**Fig. 5D**). To investigate the reason of the differential sensitivity of GTR0511 to erlotinib, we analyzed whole-exome sequencing data, but we did not detect *EGFR* alterations (data not shown). On the contrary, RNA-seq analysis revealed a 10-fold decrease of the number of reads covering the last portion of the receptor (from exon 26 until the end of the mRNA; Supplementary Fig. S4A). This resulted in the presence of an EGFR protein isoform lacking the C-terminal domain, together with an EGFR full-length protein. As Kovacs and colleagues (36) showed that the loss of this portion of the tail, containing Y1068, determines a strong decrease in receptor activation, we immunoprecipitated (with an antibody directed against the EGFR extracellular portion) EGFR from organoids derived from the three PDXs. As shown in Supplementary Fig. S4B, in GTR0511, EGFR displayed only a modest activation, in spite of the high amount of the expressed protein, meaning that the ratio between phosphorylated/unphosphorylated receptor was much lower in GTR0511 compared with the other amplified models. As predicted by *in silico* data, two phosphorylated bands were detected only in GTR0511, and they were both effectively inhibited by erlotinib. Finally, stronger downstream signal blockade in GTR0511 versus GTR0110 and GTR0060 was seen in total cell lysates derived from the same organoids. In agreement with previously published data (36), we thus hypothesize that the lack of the EGFR C-terminal tail in GTR0511 can be responsible of its decreased activation and increased sensitivity to erlotinib treatment.

To investigate which pathways were inactivated by the different drugs/drug combinations in cases in which the combination resulted in a strongly enhanced response, we took advantage of PDX-derived primary cells in which *EGFR* amplification was maintained (Supplementary Fig. S5A). Primary cells were treated with cetuximab, erlotinib, and lapatinib, alone or in combination. Western blot analysis showed that while lapatinib and erlotinib only slightly affected activation of downstream transducers, such as AKT, MAPK, and S6 (evaluated as read out of the PI3K, RAS/MAPK, and mTOR pathways, respectively), a partial inhibition was induced by cetuximab. Interestingly, both the dual combinations resulted in a strong inhibition of signal transduction (**Fig. 5E**). Phospho-array analysis of cellular kinases and RTKs confirmed these results, but did not identify any other kinase specifically inhibited by the combination treatments (Supplementary Fig. S5B). These *in vitro* data strongly support the results we obtained in the *in vivo* experiments where cetuximab induced SD, while the two combinations resulted in a complete and durable response. It is thus likely that when EGFR activation is exceptionally intense, the dual blockade with TKI + cetuximab is needed to improve the response.

TSC2 inactivation is a mechanism of resistance to EGFR-targeted therapies

We performed a preclinical trial, similar to those described previously, using the GTR0078 PDX harboring approximately 700 *EGFR* copies (Supplementary Fig. S3). Despite the very high level of *EGFR* amplification, we did not observe response to the TKIs, nor to cetuximab or cetuximab + TKI combination treatments (**Fig. 6A**). To understand the molecular basis for the observed resistance, we sequenced the tumor DNA and detected several genomic alterations; among these, we observed a fraction of *EGFR* gene copies displaying a deletion at the 5′ gene portion, thus coding for a protein lacking the extracellular portion (Supplementary Fig. S6A). Moreover, we also observed two missense *TSC2* mutations (p.M1300V and p.R1438Q), with an allelic frequency of 0.463 and 0.539, respectively (**Fig. 6B**). The TSC2 protein forms a complex with TSC1, a critical negative regulator of mTOR complex (mTORC) 1, which controls anabolic processes to promote cell growth (37–39). TSC2 inactivation (due to homozygous mutations or gene loss) results in increased mTOR activation (40). Interestingly, when we interrogated cBioPortal for the possible cooccurrence of *EGFR* and *TSC2* functional genomic alterations in six gastric cancer datasets (4, 41–45), we found a significant correlation (Supplementary Fig. S6B). Moreover, alterations in the mTOR pathway cooccurrent with *EGFR* CNG have been identified in the FMI dataset as well, although cooccurrence with *EGFR* amplification was uncommon (Supplementary Fig. S6C).

To support the causative role of TSC2 in EGFR target therapy resistance, we silenced *TSC2* in OE21 cells, harboring *EGFR* gene amplification (46). In *in vitro* experiments, upon *TSC2* silencing, we observed the constitutive activation of the mTOR pathway, revealed by the activation of the downstream transducer S6, which was maintained even in the presence of anti-EGFR treatment (Supplementary Fig. S7A). To validate these data *in vivo*, we transduced OE21 cells with either control shRNA (shC) or a pool of *TSC2* shRNAs and we injected them in immunocompromised mice. As shown in Supplementary Fig. S7B, shC mice underwent tumor regression in response to EGFR blockade, while partially *TSC2*-silenced tumors experienced only disease stabilization, reinforcing the idea that *TSC2* silencing impairs the response to anti-EGFR therapy.

We thus wondered whether treatment of GTR0078 tumors with an mTOR inhibitor (such as everolimus) could restore sensitivity to EGFR inhibitors. While treatment with everolimus alone did not show any clinical efficacy (Supplementary Fig. S6D), the combination of everolimus with erlotinib resulted in a significant clinical response (**Fig. 6C**). Experiments performed in PDX-derived cells showed that while treatment of GTR0078 cells with either EGFR inhibitors or everolimus was unable to block mTOR activation, the association of the two drugs resulted in a sustained inhibition of the pathway. Indeed, only the concomitant inhibition of the EGFR and mTOR pathway inactivated the downstream transducer S6 kinase (**Fig. 6D**).

Discussion

In unselected patients with advanced gastric/esophageal adenocarcinoma, the addition of an anti-EGFR antibody to first-line standard chemotherapy failed to show a significant survival benefit in two RCTs (8, 9). Similar negative results were also observed when the

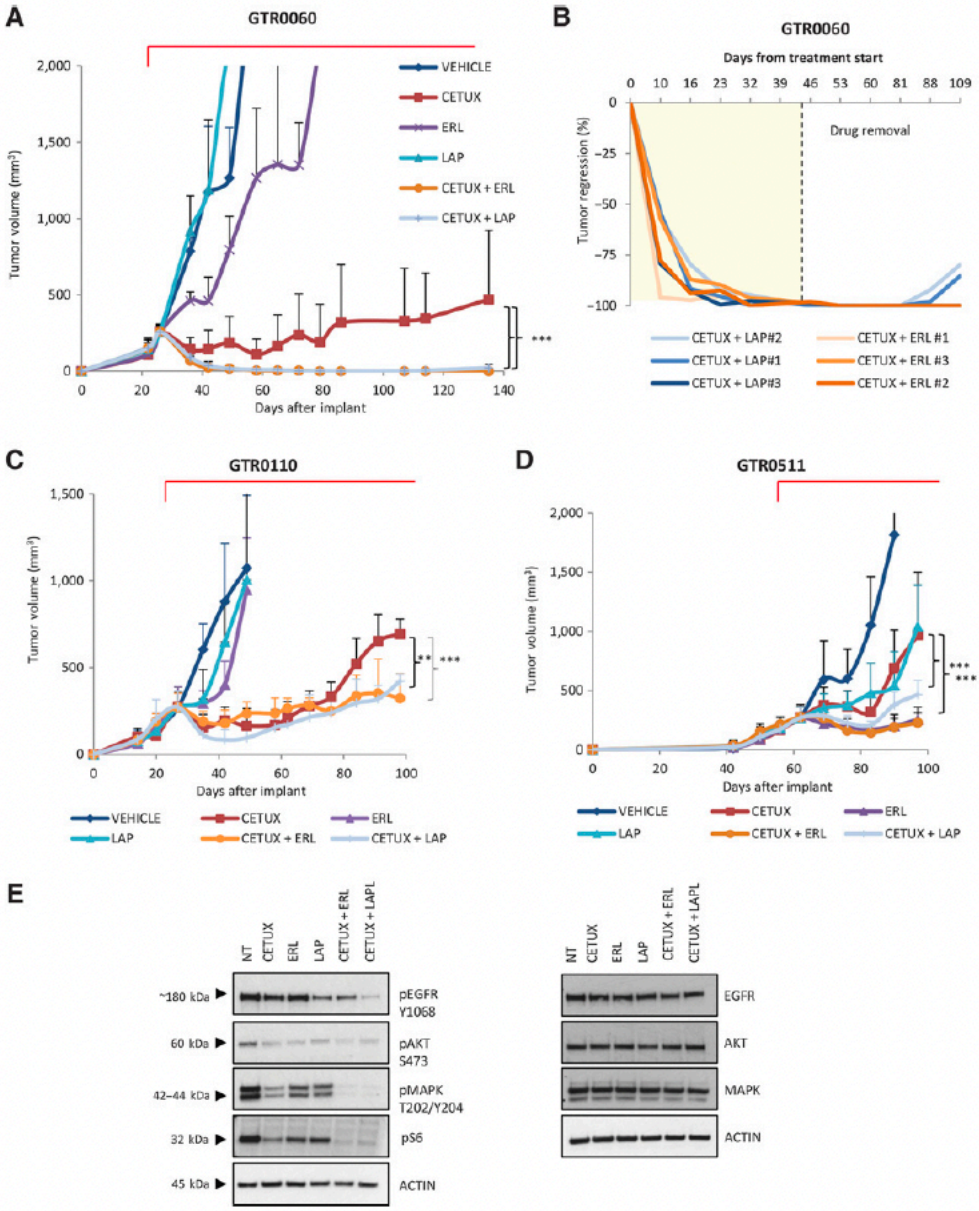
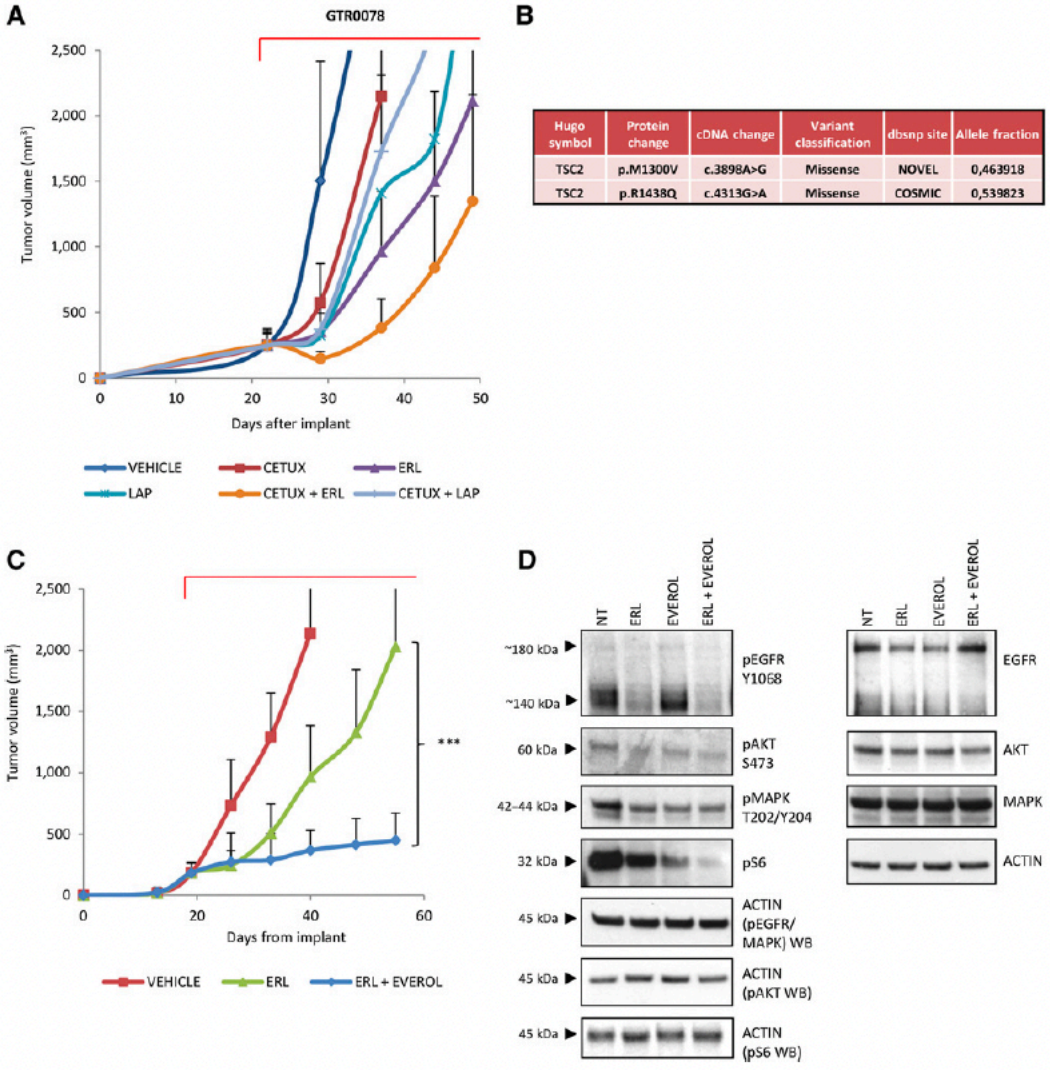


Figure 5.

Dual EGFR blockade is the most effective treatment in *EGFR*-amplified PDXs. Tumor growth curves in mice cohorts derived from GTR0060 (**A**), GTR0110 (**C**), and GTR0511 (**D**) patients treated with the EGFR inhibitors, cetuximab (CETUX), erlotinib (ERL), and lapatinib (LAP), alone or in combination, as indicated. The red lines indicate the day when treatment was started. The response in mice has been evaluated using RECIST1.1–like criteria, that is, PD: ≥35% increase from baseline; PR: ≥ 50% reduction from baseline; and SD: intermediate variations from baseline. **B,** Spaghetti plot illustrating drug response in the xenotrial performed on the cohort of mice derived from GTR0060 PDX. Individual lines represent, for each mouse, the percentage variation in tumor burden, from start of treatment (day 0). Blue lines, cetuximab + lapatinib–treated mice and red lines, cetuximab + erlotinib–treated mice. Dashed line indicates treatment stop. **E,** Western blot analysis of the activation state of EGFR and its downstream targets (AKT, MAPK, and S6) in GTR0060 tumor–derived cells treated with the indicated drugs/drug combinations. Actin was used as loading control. Statistical significance is indicated (**, P <0.01; ***, P < 0.001).



Hugo symbol	Protein change	cDNA change	Variant classification	dbsnp site	Allele fraction
TSC2	p.M1300V	c.3898A>G	Missense	NOVEL	0,463918
TSC2	p.R1438Q	c.4313G>A	Missense	COSMIC	0,539823

Figure 6.

TSC2 inactivation is a mechanism of resistance to EGFR-targeted therapies. **A,** Tumor growth curves in the mice cohorts derived from GTR0078 and treated with the EGFR inhibitors, cetuximab (CETUX), erlotinib (ERL), and lapatinib (LAP), alone or in combination, as indicated. The red line indicates the day when treatment was started. **B,** The table shows the two *TSC2* mutations identified in GTR0078 PDX. **C,** Tumor growth curves in the mice cohorts derived from GTR0078 and treated with erlotinib or the combination erlotinib + everolimus (ERL+ EVEROL). The red line indicates the day when treatment was started. **D,** Western blot analysis of the activation state of EGFR and its downstream targets (AKT, MAPK, and S6) in GTR0078 tumor–derived cells treated with the indicated drugs/drug combinations. Actin was used as loading control. Statistical significance is indicated (***, $P < 0.001$).

small-molecule TKI, gefitinib, was compared with placebo from the second-line setting and beyond (10). Sporadic responses to EGFR inhibitors observed in these trials, however, led several researchers to postulate the existence of a subset of metastatic patients with *EGFR*-addicted tumors, potentially vulnerable to EGFR blockade (13). The amplification of the *EGFR* gene is found in 3%–5% of primary gastroesophageal adenocarcinoma tumors (4, 6) and highly correlates with poor prognosis (47). By exploiting four different datasets, we have shown here that *EGFR* amplification has similar prevalence and is associated with poorer survival in the metastatic setting. This was also confirmed in the nonmetastatic setting, by analyzing TCGA data. In a prespecified exploratory analysis of one of those datasets, the COG trial, randomizing 209 chemoresistant metastatic patients to gefitinib or placebo (10), found that *EGFR* amplification was a positive

predictive marker for EGFR targeting, whereas a smaller advantage was observed in patients with chromosome 7 polysomy (13). Response to the anti-EGFR mAb, cetuximab, used alone or in combination with chemotherapy, was reported in a small set of 7 *EGFR*-amplified patients; albeit the role of the cytotoxic backbone contribution cannot be ruled out in three responders, one response was induced by EGFR blockade alone (48). Such results clearly mirror those achieved in patients receiving panitumumab monotherapy by our study. All together, these observations suggest that *EGFR* is an oncogenic driver, with potentially exquisite sensitivity to EGFR-targeting drugs, in a small but clinically consistent subgroup of gastroesophageal adenocarcinomas. On the other hand, in these *EGFR*-amplified tumors, we observed the presence of selected cooccurring driver alterations. Specifically, *MET/HER2/KRAS* coamplifications and *KRAS/PIK3CA/PTEN* comutations were identified in 53% and 35% of patients in the *EGFR*-amplified subgroups included in TCGA and the FMI datasets, respectively; this result highlights that only a subset of patients with *EGFR*-amplified gastroesophageal cancer may significantly benefit from single-agent anti-EGFR therapy. Here, we have, for the first time, functionally identified *TSC2* mutations as a potential new mechanism conferring resistance to EGFR inhibition in gastroesophageal adenocarcinomas. TSC2 is a GTPase-activating protein, whose loss or inactivating mutation results in the constitutive load of Rheb with GTP and activation of mTORC signaling (39). Interestingly, according to cBioPortal, *TSC1/TSC2* mutations are significantly associated with *EGFR* amplification (but not with other RTKs) in gastroesophageal adenocarcinomas, possibly indicating that mTORC constitutive activation can sustain the oncogenic role of *EGFR*. Our preclinical trial in an *EGFR*-amplified/*TSC2*-mutated gastroesophageal adenocarcinoma PDX confirms this hypothesis. The pharmacologic inhibition of TSC2-sustained mTORC activation by everolimus, a clinical-grade small-molecule mTOR inhibitor, overcame primary resistance and restored sensitivity to EGFR inhibition. Our data are reinforced by a recently published article from Arteaga and colleagues (49), in which they showed that hyperactivation of the mTORC pathway drives resistance to therapies targeting another member of the HER family, namely HER2, in *HER2*-mutant breast cancer. In their work, similarly to what we have observed, the combination of the TORC1 inhibitor, everolimus, and neratinib overcame resistance.

Resistance is a common occurrence of RTK inhibition across diseases, targets, and drugs. Several cell autonomous mechanisms sustaining resistance to driver RTKs have been identified so far, including mutations of the target itself, activation of downstream transducers, activation of parallel pathways, and transdifferentiation. Moreover, in many cases, the amplified RTK is not located in the natural genomic site, but it is rather extrachromosomal. This results in a mechanism favoring rapid adaptation of cancer cells to environmental changes. Indeed, as extrachromosomal DNA lacks centromeres, it is unequally segregated during cell division, leading to increased tumor heterogeneity and different cellular fitness in diverse contexts. Cancer cells in which oncogenes are extrachromosomal can thus become resistant to RTK inhibitors either by increasing the number of gene copies (thus titrating the amount of the available inhibitor) or by progressively decreasing the number of gene copies. Both the mechanisms are sustained by experimental data. For example, Nathanson and colleagues (50) showed that glioblastoma cells can become resistant to erlotinib by eliminating extrachromosomal copies of the mutant *EGFR* gene. This "adaptation" to the treatment can be acquired and expanded along tumor evolution, enabling tumors to maintain their intratumoral heterogeneity. In previous works (51, 52), we have shown that in *MET*-hyperamplified gastric cancer cells (where the amplified gene was extrachromosomal), resistance was due to further acquisition of gene copies; this resulted in an amount of activated receptor overcoming the inhibitory ability of the drugs at tolerable doses.

To bypass primary and prevent secondary resistance to EGFR-targeted drugs in *EGFR*-amplified gastroesophageal adenocarcinomas, we leveraged our large platform of 151 primary gastroesophageal adenocarcinomas patient–derived mouse avatars (22), enriched for 15 cases with *EGFR* gene copy gain, including four avatars with more than eight *EGFR* copies (confirmed as amplified, i.e., nonpolysomic, by silver ISH). EGFR inhibition, in absence of chemotherapy, resulted in a clinical response in three of four cases. Notably, one of these cases featured 12 *EGFR* copies, a range of amplification that is just above the threshold (eight copies) considered biologically relevant and that has not been investigated previously (48). Interestingly, a CR was achieved only in the PDX with the highest *EGFR* CNG, suggesting that a higher level of gene amplification may be associated with a greater magnitude of treatment benefit, as it is known for *HER2*-amplified gastroesophageal adenocarcinoma and breast cancer (30, 53).

The pharmacologic space of EGFR-targeted drugs is well populated by antibodies and small-molecule TKIs, both experimental and approved for use in clinically diverse settings (54, 55).

In our preclinical trials in *EGFR*-amplified gastroesophageal adenocarcinoma avatars, we compared the efficacy of randomly allocated TKIs and cetuximab, delivered as single agent or in combinations. Erlotinib and cetuximab showed single-agent excellent activity in one and two models, respectively, while in a third model, cetuximab treatment resulted in disease stabilization. Importantly, however, the dual EGFR blockade resulted in a sustained significant response in all three models, suggesting that a strong inhibition of the downstream transducers is needed to eradicate the disease.

In conclusion, our study further corroborates *EGFR* amplification as an actionable therapeutic target in gastroesophageal adenocarcinoma, demonstrates that a dual EGFR blockade may be needed to maximize the therapeutic efficacy, and identifies potential mechanisms of primary resistance, specifically the mTORC pathway, paving the way for experimentally driven clinical trials. In fact, the next-generation clinical trial landscape in *EGFR*-amplified gastroesophageal adenocarcinomas may not be at a dead end. The combination of lapatinib and cetuximab has already been proven safe in a phase I trial (56), potent second-generation antibodies mixtures against different, non-overlapping epitopes of EGFR, such as Sym004 and MM-151 (57, 58), are into clinical development, and the TORC pathway is targetable with commercially available drugs. Given the diversity of clinically relevant genomic alterations and lack of benefit from EGFR-targeted therapies in unselected gastroesophageal adenocarcinoma populations, broad-based genomic profiling is thus necessary to reliably detect *EGFR* gene amplification in addition to other potential drivers and mechanisms of resistance.

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Authors' Contributions

S. Corso: Conceptualization, formal analysis, supervision, funding acquisition, writing–original draft, writing–review and editing. **F. Pietrantonio:** Conceptualization, resources, supervision, funding acquisition, writing–original draft, writing–review and editing. **M. Apicella:** Conceptualization, resources, supervision, investigation, writing–original draft, writing–review and editing. **C. Migliore:** Resources, data curation, formal analysis, investigation, writing–review and editing. **D. Conticelli:** Resources, formal analysis, investigation, writing–review and editing. **A. Petrelli:** Resources, formal analysis, validation, investigation, writing–review and editing. **L. D'Errico:** Resources, validation, investigation, writing–review and editing. **S. Durando:** Resources, validation, investigation, writing–review and editing. **D. Moya-Rull:** Resources, data curation, formal analysis, validation, investigation, writing–review and editing. **S.E. Bellomo:** Resources, data curation, formal analysis, validation, investigation, writing–review and editing. **S. Ughetto:** Resources, data curation, validation, investigation, writing–review and editing. **M. Degiuli:** Resources, data curation, writing–review and editing. **R. Reddavid:** Resources, data curation, writing–review and editing. **U. Fumagalli:** Resources, data curation, writing–review and editing. **S. De Pascale:** Resources, data curation, writing–review and editing. **G. Sgroi:** Resources, data curation, writing–review and editing. **E. Rausa:** Resources, data curation, writing–review and editing. **G.L. Baiocchi:** Resources, data curation, writing–review and editing. **S. Molfino:** Resources, data curation, writing–review and editing. **G. De Manzoni:** Resources, data curation, writing–review and editing. **M. Bencivenga:** Resources, data curation, writing–review and editing. **S. Siena:** Resources, data curation, writing–review and editing. **A. Sartore-Bianchi:** Resources, data curation, formal analysis, visualization, writing–review and editing. **F. Morano:** Resources, data curation, formal analysis, visualization, writing–review and editing. **S. Corallo:** Resources, data curation, visualization, writing–review and editing. **M. Prisciandaro:** Resources, data curation, visualization, writing–review and editing. **M. Di Bartolomeo:** Data curation, formal analysis, investigation, writing–review and editing. **A. Gloghini:** Formal analysis, investigation, project administration, writing–review and editing. **S. Marsoni:** Methodology, project administration, writing–review and editing. **A. Sottile:** Formal analysis, funding acquisition, methodology, writing–review and editing. **A. Sapino:** Resources, formal analysis, funding acquisition, writing–review and editing. **C. Marchiò:** Resources, data curation, formal analysis, writing–review and editing. **A. Dahle-Smith:** Data curation, writing–review and editing. **Z. Miedzybrodzka:** Data curation, writing–review and editing. **J. Lee:** Data curation, writing–review and editing. **S.M. Ali:** Data curation, writing–review and editing. **J.S. Ross:** Data curation, writing–review and editing. **B.M. Alexander:** Data curation, writing–review and editing. **V.A. Miller:** Data curation, writing–review and editing. **R. Petty:** Data curation, writing–review and editing. **A.B. Schrock:** Conceptualization, data curation, supervision, funding acquisition, writing–original draft, project administration, writing–review and editing. **S. Giordano:** Conceptualization, resources, formal analysis, supervision, funding acquisition, investigation, writing–original draft, project administration, writing–review and editing.

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cancers

MDPI

Systematic Review

Molecularly Targeted Therapies for Gastric Cancer. State of the Art

Rossella Reddavid [1,2], Simona Dagatti [1,2], Caterina Franco [1,2], Lucia Puca [1,2], Mariano Tomatis [1,2], Simona Corso [3,4], Silvia Giordano [3,4] and Maurizio Degiuli [1,*]

[1] Department of Oncology, Università degli Studi di Torino, 10126 Torino, Italy; rossella.reddavid@unito.it (R.R.); simona.dagatti@unito.it (S.D.); caterinafranco@hotmail.it (C.F.); lucia.pucamed@gmail.com (L.P.); mariano.tomatis@gmail.com (M.T.)
[2] Surgical Oncology and Digestive Surgery Unit, San Luigi University Hospital, Regione Gonzole 10, Orbassano, 10043 Turin, Italy
[3] Department of Oncology, University of Torino, 10060 Candiolo, Italy; simona.corso@ircc.it (S.C.); silvia.giordano@ircc.it (S.G.)
[4] Candiolo Cancer Institute, FPO-IRCCS, Strada Provinciale 142, Candiolo, 10060 Turin, Italy
* Correspondence: maurizio.degiuli@unito.it; Tel.: +39-335-8111286

Simple Summary: Despite recent advances in surgical techniques and in anticancer drugs, and the adoption of perioperative treatments mostly based on conventional chemotherapy, the prognosis of advanced and metastatic gastric cancer remains poor. In the last decade, the addition of molecular therapy did not show any significant survival advantage, and the first reports available documented an increase of the rate of severe adverse effects and related mortality. We conducted a literature search for randomized trials investigating novel molecular agents as compared to conventional chemotherapy. The outcomes were patients' survival and the rates of tumor response and of severe adverse effects (SAE). Although we did not find an increase of SAE, the survival benefits of novel molecular therapies available to date for advanced and metastatic gastric cancer were rather unclear, mostly due to inaccurate patient selection, particularly concerning oncogene amplification and copy number.

Abstract: Many phase III trials failed to demonstrate a survival benefit from the addition of molecular therapy to conventional chemotherapy for advanced and metastatic gastric cancer, and only three agents were approved by the FDA. We examined the efficacy and safety of novel drugs recently investigated. PubMed, Embase and Cochrane Library were searched for phase III randomized controlled trials published from January 2016 to December 2020. Patients in the experimental arm received molecular therapy with or without conventional chemotherapy, while those in the control arm had conventional chemotherapy alone. The primary outcomes were overall and progression-free survival. The secondary outcomes were the rate of tumor response, severe adverse effects, and quality of life. Eight studies with a total of 4223 enrolled patients were included. The overall and progression-free survival of molecular and conventional therapy were comparable. Most of these trials did not find a significant difference in tumor response rate and in the number of severe adverse effects and related deaths between the experimental and control arms. The survival benefits of molecular therapies available to date for advanced and metastatic gastric cancer are rather unclear, mostly due to inaccurate patient selection, particularly concerning oncogene amplification and copy number.

Keywords: gastric cancer; molecular target therapy; chemotherapy; EGFR inhibitors; angiogenesis inhibitors; MET inhibitors

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1. Introduction

Gastric cancer is one of the most frequent malignancies. It represents the fifth most frequent cancer worldwide (5.6%) and the fourth leading cause of cancer-related death (7.7%) with 768,793 deaths per year in 2020 [1].

Surgical resection with optimal lymphadenectomy is the only curative treatment in cases of AGC [2–6]. In recent decades, several perioperative and postoperative regimens of conventional CT have been investigated, and neoadjuvant treatment has been recommended as mandatory in several national guidelines, but the prognosis of stage III and IV GC remains poor [7–10]. In 2014, Cancer Genome Atlas Research Network paved the way for a new molecular classification of GC and documented the existence of four subtypes: EBV (9%), MSI (22%), CIN (50%), and GS (20%) [11]. The identification of these subtypes and the related signaling pathways provided a roadmap for GC patient stratification and promising strategies for targeted therapies. Trastuzumab was the first MT approved by the FDA and European Union for AGC; it was subsequently introduced as the standard of care for patients with locally or fAGC displaying HER2 overexpression/amplification [12]. In 2014, the FDA also approved the use of ramucirumab as monotherapy or in combination with paclitaxel for advanced and metastatic GC [13]. To date, only these two MTs (in addition to the antibody–drug conjugate trastuzumab deruxtecan) have been approved, although many other molecular targets have been identified in recent years. Indeed, the majority of phase III trials investigating novel molecular agents failed to demonstrate their efficacy, mostly due to inaccurate patient selection (particularly concerning driver gene amplification and copy number) and the lack of preclinical models supporting proof of concepts followed by structured trials. PDXs are helpful in validating and predicting the response to novel MTs, even though these models are unable to reproduce the same conditions and environmental characteristics of the donor tumor and very rarely allow metastatic dissemination [14]. For this purpose, PDOXs were recently introduced in GC preclinical research to better recapitulate the original cancer background [15].

In 2016, the Cochrane Collaborative Group published a systematic review with the aim of assessing the efficacy and safety of MTs available for the treatment of advanced and metastatic gastric cancer [16]. The authors identified 11 RCTs enrolling a total of 4014 patients with AGC who underwent conventional CT and MT or conventional CT alone. They concluded that the benefit of MTs on survival was unclear and pointed out a significant increase in side effects.

The present systematic review and meta-analysis aims to examine the efficacy and safety of novel MTs investigated in the years after publication of the Cochrane review.

2. Molecular Targets and Target Agents

2.1. Epidermal Growth Factor Receptor

EGFRs include four types of TKRs (HER1/EGFR, HER2, HER3, HER4) located on the cell surface. They play an important role, conveying messages to manage cell growth and differentiation.

2.1.1. Anti-HER1

Many authors have demonstrated that approximately 30% of GCs show HER1 overexpression [17,18]. Two main monoclonal antibodies (cetuximab and panitumumab) that reduce HER1 activity by binding its extracellular domain have been identified. Moreover, cetuximab can stimulate the activity of the immune system against tumor cells [19]. Unfortunately, the heterogeneity of GC seems to affect the efficacy of cetuximab in most of these patients [20].

Gefitinib and erlotinib, two tyrosine kinase inhibitors, can also inactivate HER1 by binding its intracellular domain and blocking its kinase activity [21]. Unfortunately, phase II trials have shown that these therapies have limited efficacy [22,23]. Recently, Maron et al. and Corso et al. identified a subpopulation of GC patients presenting a high level of EGFR amplification, which is responsive to anti-EGFR drugs [24,25]. They also identified mechanisms of resistance to EGFR-targeted drugs, such as TKR activation, KRAS mutation/amplification, and TSC2 inactivation [25].

2.1.2. Anti HER2

Several authors have shown a direct relationship between HER2 amplification (and the consequent overexpression of its receptor) and many types of tumors [26]. The HER2 gene is a proto-oncogene located on chromosome 17q21. The first drug binding HER2 was trastuzumab. In 2010, the ToGa trial documented the superiority of trastuzumab in combination with conventional chemotherapy compared with chemotherapy alone in terms of OS and DFS for patients with AGC [12]. Nevertheless, only a few patients with GC (less than 20%) gain a real advantage from trastuzumab.

In the past decade, several other anti-HER2 agents have been tested for GC treatment. Lapatinib is a dual kinase inhibitor that acts on EGFR (ErbB1) and HER2 (ErbB2) with the consequent downregulation of HER2 signaling [27].

Pertuzumab is an anti-HER2 monoclonal antibody that prevents heterodimerization between HER2 and other HER family members [28].

The efficacy of the combination of trastuzumab and pertuzumab has been investigated in the JACOB trial [29]. Despite the suggestion of treatment activity (a trend towards therapeutic activity for increasing PFS and the proportion of patients who achieved an objective response), adding pertuzumab to trastuzumab and chemotherapy did not significantly improve OS in patients with HER2-positive GC vs. placebo. However, a recent preclinical trial demonstrated that a subgroup of patients with hyperamplified (>8 gene copies) HER2 could strongly benefit from dual HER2 blockade therapy [30].

T-DM1 is an antibody–drug conjugate generated by the conjugation of trastuzumab and DM1, a tubulin inhibitor [31]. The action of this drug is characterized by two phases: first, the ADC ligates the extracellular domain of HER2; it is subsequently transferred intracellularly, releasing DM1 that proceeds to block microtubule polymerization. The GATSBY trial, a randomized, open-label, adaptive, phase II/III study investigating the efficacy of T-DM1 compared to taxane in patients with previously treated, HER2-positive AGC, has just been completed and will be analyzed in this review [32].

Trastuzumab deruxtecan (DS-8201) is an antibody–drug conjugate consisting of trastuzumab, a cleavable linker, and a cytotoxic topoisomerase I inhibitor. An open-label, randomized, phase II trial performed on HER2+ GC patients evaluated trastuzumab deruxtecan vs. chemotherapy and showed that treatment with trastuzumab deruxtecan led to significant improvements in response and OS compared with standard therapies [33].

2.2. *Vascular Endothelial Growth Factor*

VEGFs are proteins promoting blood vessel formation. Four types of VEGF (VEGF-A, VEGF-B, VEGF-C, and VEGF-D) have been identified, with three types of corresponding receptors (VEGFR-1, VEGFR-2, and VEGFR-3). Several studies have reported the fundamental role of these signaling proteins in new blood vessel formation and cancer cell proliferation [34]. Furthermore, VEGF expression has been found in approximately 40% of GC [35]. Bevacizumab is an anti-VEGF-A monoclonal antibody that inhibits circulating VEGF-A activity [36]. The efficacy of this monoclonal antibody has been widely documented in several solid tumor treatments [37–39] but bevacizumab is still under investigation for its benefit in GC. Some phase II/III trials proved its efficacy in association with conventional chemotherapy in AGC, while others did not report any clear benefits [40,41]. Furthermore, Shah et al. reported improved oncologic outcomes only in Caucasian patients compared to Asian patients, suggesting that the VEGF-A pathway in GC could be different among races [42].

Many trials have investigated the efficacy of VEGF TKR inhibitors (sunitinib and sorafenib), but no phase III trial has shown any survival benefits [43,44]. Finally, a monoclonal antibody blocking VEGFR-2 was successfully introduced for advanced solid malignancy treatment in 2010 (ramucirumab) [45]. A significant improvement in survival outcomes in patients with AGC submitted to second-line therapy with ramucirumab alone or in combination with paclitaxel was documented in two main phase III trials [46,47]. Interestingly,

these two trials also highlighted significant differences in the VEGF-A pathway between Asian and non-Asian patients.

2.3. Mammalian Target of Rapamycin

mTOR is a serine/threonine protein kinase identified in mammalian cells with a leading role in controlling mechanisms of cell growth and proliferation. Human cancers can be characterized by hyperactivity or inactivity of the mTOR pathway, which plays a crucial role in maintaining tumor-modified phenotypes [48]. In 2008, Cejka et al. [49] demonstrated in vitro the efficacy of everolimus (RAD001) in inhibiting mTOR complex 1 (mTORC1, mTOR combined with the adaptor protein raptor) with consequent blockage of HIF-1α and VEGF. The authors concluded that everolimus, through the inhibition of mTORC1 in GC cells, could affect cancer proliferation and generate central tumor necrosis. Moreover, everolimus antitumor action is amplified by its association with metronomic cyclophosphamide.

2.4. Hepatocyte Growth Factor Receptor

HGFR, also known as c-MET, is a proto-oncogenic receptor tyrosine kinase that, after binding to hepatocyte growth factor, induces cell migration and proliferation, promotes mitosis, and inhibits apoptosis. C-MET overexpression and gene amplification are related to a poor prognosis [50,51].

Crizotinib (PF-02341066) is a tyrosine kinase inhibitor of the c-MET receptor and of the TKR anaplastic lymphoma kinase; it has been approved by the FDA for treatment of ALK-positive NSCLC patients. Okamoto et al. in 2012 stated that crizotinib "has pronounced effects on signal transduction and survival in gastric cancer cells with MET amplification" [52]. Phase II/III trials to evaluate crizotinib efficacy and safety in GC are ongoing.

Another promising agent targeting the HGF-cMET complex is rilotumumab. This human monoclonal antibody impairs the c-MET signaling pathway by binding to and inactivating its ligand HGF [53]. Clinical trials of this drug in GC (including two phase III trials) were halted due to a significant increase in mortality in the experimental arm (rilotumumab in combination with conventional chemotherapy) in one of these trials, but new investigations have begun.

Finally, ornatuzumab is a humanized monoclonal antibody that binds to the extracellular receptor of c-MET, counteracting its activation by HGF ligand [54]. METGastric, a phase III trial of onartuzumab plus standard first-line chemotherapy for HER2, was recently conducted in MET+ advanced GC. Results of this study will be discussed in this review.

Table 1 summarizes the disappointing results of phase II and III trials that target HER2, EGFR, VEGF, VEGFR, MET, mTOR, and others.

Table 1. Results of phase II and III trials. This table summarizes recent phase II and III RCTs investigating novel molecular agents' survival outcomes. Unfortunately, most of these trials did not show any overall and progression free survival advantages as compared to conventional chemotherapy (red dot). Positive and partially positive studies have been pointed out with green and orange dot, respectively.

Trial, Year	EXP Arm	CTR Arm	Molecular Target	Nr Total Pts (EXP/CTR)	Treatment Line	Phase	Median OS (Months)	Median PFS (Months)	Results
REAL-3 [55], 2009	EOC + Panitumumab	EOC	EGFR	553	I	III	11.3 CTR arm 8.8 EXP arm 95%CI: 1.07–1.76 $p = 0.013$ HR = 1.37	7.4 CTR arm 6.0 EXP arm 95%CI: 0.98–1.52 $p = 0.068$ HR = 1.22	🔴
AVAGAST [40], 2012	XP + Bevacizumab	XP + Placebo	VEGF	774	I	III	10.1 CTR arm 12.1 EXP arm 95%CI: 0.73–1.03 $p = 0.1002$ HR = 0.87	5.3 CTR arm 6.7 EXP arm 95%CI: 0.68–0.93 $p = 0.0037$ HR = 0.80	🟠
FAST [56], 2012	EOX + Claudiximab	EOX	Claudin 18.2	161	I	II	8.4 CTR arm 13.4 EXP arm 95%CI: 0.36–0.73 $p < 0.001$ HR = 0.51	4.8 CTR arm 7.9 EXP arm 95%CI: 0.31–0.70 $p = 0.0001$ HR = 0.47	🟢
INTEGRATE [57], 2012	Regorafenib	Placebo	VEGF, RET, RAF	147	II III	II	4.5 CTR arm 5.3 EXP arm 95%CI: 0.51–1.08 $p = 0.147$ HR = 0.74	0.9 CTR arm 2.6 EXP arm 95%CI: 0.28–0.59 $p < 0.001$ HR = 0.4	🟠
ENRICH (NCT01813253), 2013	Irinotecan + Nimotuzumab	Irinotecan	EGFR	400	II	III	NO RESULT POSTED	NO RESULT POSTED	🔴
LOGiC [58], 2013	XELOX + Lapatinib	XELOX + Placebo	HER2	545	I	III	10.5 CTR arm 12.2 EXP arm 95%CI: 0.73–1.12 $p = 0.3492$ HR = 0.91	5.4 CTR arm 6.0 EXP arm 95%CI: 0.68–1 $p = 0.0381$ HR = 0.82	🔴
JapicCTI-090849 [59], 2014	Irinotecan + Nimotuzumab	Irinotecan	EGFR	83	II	II	7.7 CTR arm 8.4 EXP arm 95%CI: 0.618–1.599 $p = 0.9778$ HR = 0.994	2.9 CTR arm 2.4 EXP arm 95%CI: 0.516–1.435 $p = 0.5668$ HR = 0.860	🔴

Table 1. *Cont.*

Trial, Year	EXP Arm	CTR Arm	Molecular Target	Nr Total Pts (EXP/CTR)	Treatment Line	Phase	Median OS (Months)	Median PFS (Months)	Results
RAINBOW [47], 2014	Paclitaxel + Ramucirumab	Paclitaxel + Placebo	VEGFR2	665	II	III	7.36 CTR arm 9.63 EXP arm 95%CI: 0.678–0.962 $p = 0.0169$ HR = 0.807	2.86 CTR arm 4.4 EXP arm 95%CI: 0.536–0.752 $p < 0.0001$ HR = 0.635	🟢
REGARD [46], 2014	Ramucirumab	Placebo	VEGFR2	355	II	III	3.8 CTR arm 5.2 EXP arm 95%CI: 0.603–0.998 $p = 0.047$ HR = 0.776	1.3 CTR arm 2.1 EXP arm 95%CI: 0.376–0.620 $p < 0.0001$ HR = 0.483	🟢
ToGA [12], 2014	FP/XP + Trastuzumab	FP/XP	HER2	594	I	III	11.1 CTR arm 13.8 EXP arm 95%CI: 0.60–0.91 $p = 0.0046$ HR = 0.74	5.5 CTR arm 6.7 EXP arm 95%CI: 0.59–0.85 $p = 0.0002$ HR = 0.71	🟢
TyTAN [27], 2014	PTX + Lapatinib	PTX	HER2	261	II	III	8.9 CTR arm 11 EXP arm 95%CI: 0.64–1.11 $p = 0.1044$ HR = 0.84	4.4 CTR arm 5.4 EXP arm 95%CI: 0.63–1.13 $p = 0.2241$ HR = 0.85	🔴
GRANITE-1 [60], 2015	Everolimus	Placebo	mTOR	656	II III	III	4.34 CTR arm 5.39 EXP arm 95%CI: 0.75–1.08 $p = 0.1244$ HR = 0.90	1.41 CTR arm 1.68 EXP arm 95%CI: 0.56–0.78 $p < 0.0001$ HR = 0.66	🔴
EXPAND [61], 2016	XP + Cetuximab	XP	EGFR	904	I	III	10.7 CTR arm 9.4 EXP arm 95%CI: 0.87–1.17 $p = 0.95$ HR = 1	5.6 CTR arm 4.4 EXP arm 95%CI: 0.92–1.29 $p = 0.32$ HR = 1.09	🔴

Nr: number; pts: patients; OS: overall survival; PFS: progression free survival; EXP: experimental; CTR: control; XELOX: capecitabine and oxaliplatin; EOC/EOX: epirubicin + oxaliplatin + capecitabine, XP: capecitabine and Cisplatin, FP: 5-fluorouracil and cisplatin, PTX: paclitaxel, CI: confidence interval, HR: hazard ratio. 🟢 positive study. 🟠 partially positive study. 🔴 negative study.

In Figure 1, targeted therapies and oncogenic pathways in gastric cancer are detailed.

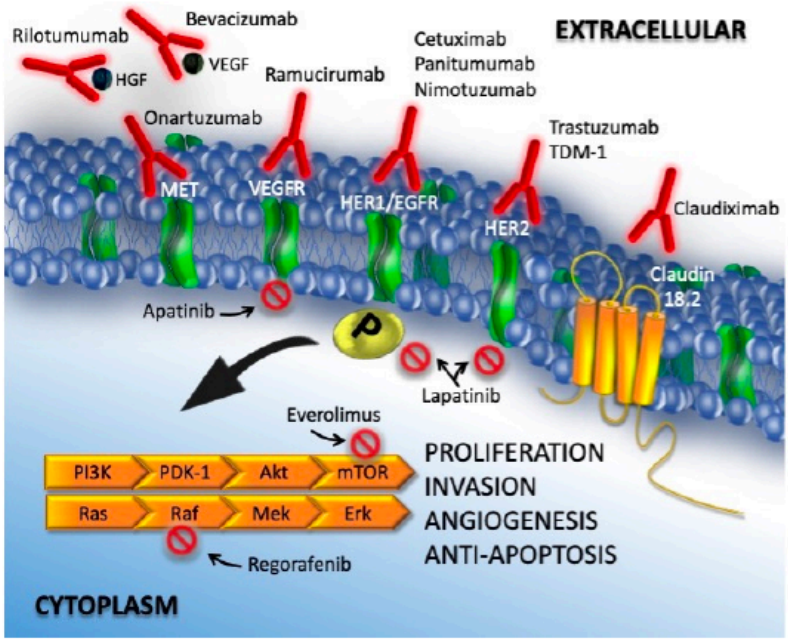


Figure 1. Targeted therapy and oncogenic pathways in gastric cancer. *Activation of ERK-AMP KINASE*: ligand binding to a growth factor receptor activates the small GTP-binding RAS protein, which interacts with RAF protein kinase. RAF phosphorylates and activates MEK (MAP kinase or ERK kinase), which then activates ERK (extracellular signal-regulated kinase) by phosphorylation of tyrosine and threonine residues. Activated ERK translocates into the nucleus where it phosphorylates the Elk-1 transcription. *PI3K/AKT/MTOR Pathway*: PI3K/AKT/MTOR signaling constitutes an important pathway that consists of two steps: phosphatidylinositol 3-kinase (PI3K) and its downstream molecule serine/threonine protein kinase B (PKB; also known as AKT). The PI3/AKT/mTOR pathway is stimulated by RTK and cytokine receptor activation. Tyrosine residues are then phosphorylated and provide anchor sites for PI3K translocation to the membrane, thus participating in the transduction of various extracellular matrix molecules and cytokines, including mTOR, a serine/threonine protein kinase and a member of the PI3K-associated kinase protein family.

2.5. Preclinical Trials

Preclinical trials have proved to be valuable tools to derive molecular information to better target GC for innovative MTs and stratify patients for clinical trials. The use of organoids, PDXs, and PDOXs in GC research showed interesting patient related tumor characteristics and cancer escape mechanisms. Several authors reported a strong relationship between higher levels of HER2 amplification/copy number and increased benefit of Trastuzumab in AGC [30,62]. More recently, a preclinical trial on PDXs allowed a TSC2 mutation leading to increased resistance to EGFR inhibition to be identified. The pharmacological inhibition of TSC2 was positively tested with everolimus, which was able to overcome the resistance and to reestablish the sensitivity to EGFR inhibition [25].

3. Materials and Methods

3.1. Inclusion and Exclusion Criteria

The articles included in this systematic review and meta-analysis were phase III RCTs with available abstracts and full texts in English. In the experimental arm of the trial, patients received a molecular agent with or without conventional CT, while in the control

arm, they received a placebo or conventional CT alone. Trials containing immunotherapy were not considered.

Reviews, meta-analyses, letters to the editor, editorials, case reports, retrospective studies, and conference abstracts were excluded.

Only RCTs recruiting adult patients (>18 years) with histologically proven gastric adenocarcinoma, with or without metastasis, were included in this study.

3.2. Outcomes

The primary outcomes of this meta-analysis were OS and PFS.

The secondary outcomes were overall response rate according to RECIST criteria, QoL, and side effects evaluated with specific scores [63,64].

3.3. Search Strategy

A computerized literature search of PubMed, Embase, and the Cochrane Library Central Register of Controlled Trials databases was conducted in December 2020 covering a period from 1/1/2016 to 9/12/2020, using combinations of free-text words and Medical Subject Headings (MeSH)/EMTREE terms: ("Stomach Neoplasms"[Mesh] OR ((stomach[tiab] OR gastric[tiab] OR esophago-gastr*[tiab] OR gastro-esophag*[tiab] OR gastroesophag*[tiab] OR oesophagogastr*[tiab] OR oesophago-gastr*[tiab] OR gastro-oesophag*[tiab]) AND (cancer*[tiab] OR tumor*[tiab] OR tumour*[tiab] OR neoplas*[tiab] OR carcinoma*[tiab] OR adenocarcinoma*[tiab] OR malignan*[tiab]))) AND ("Molecular Targeted Therapy"[Mesh] OR targeted-therap*[tiab] OR targeting-therap*[tiab] OR target-therap*[tiab] OR therapy-targeting[tiab] OR therapies-targeting[tiab] OR targeted-molecular[tiab] OR target-molecular[tiab] OR molecular-therap*[tiab] OR "Antibodies, Monoclonal"[Mesh] OR trastuzumab[tiab] OR "Lapatinib"[Mesh] OR lapatinib[tiab] OR cetuximab[tiab] OR panitumumab[tiab] OR "nimotuzumab"[Supplementary Concept] OR nimotuzumab[tiab] OR bevacizumab[tiab] OR "ramucirumab"[Supplementary Concept] OR ramucirumab[tiab] OR "apatinib"[Supplementary Concept] OR apatinib[tiab] OR "regorafenib"[Supplementary Concept] OR regorafenib[tiab] OR "rilotumumab"[Supplementary Concept] OR rilotumumab[tiab] OR "onartuzumab"[Supplementary Concept] OR onartuzumab[tiab] OR "Everolimus"[Mesh] OR everolimus[tiab] OR "zolbetuximab"[Supplementary Concept] OR claudiximab[tiab] OR zolbetuximab[tiab] OR "andecaliximab"[Supplementary Concept] OR andecaliximab[tiab] OR "Erlotinib Hydrochloride"[Mesh] OR erlotinib[tiab] OR "Gefitinib"[Mesh] OR gefitinib[tiab] OR"Sunitinib"[Mesh] OR sunitinib[tiab] OR "Sorafenib"[Mesh] OR sorafenib[tiab] OR "cediranib"[Supplementary Concept] OR cediranib[tiab] OR "GSK 1363089"[Supplementary Concept] OR foretinib[tiab] OR "Crizotinib"[Mesh] OR crizotinib[tiab] OR "marimastat"[Supplementary Concept] OR marimastat[tiab] OR prinostat[tiab] OR "AZD4547"[Supplementary Concept] OR AZD4547[tiab] OR AZD-4547[tiab] OR "brivanib"[Supplementary Concept] OR brivanib[tiab] OR "Vorinostat"[Mesh] OR vorinostat[tiab] OR "catumaxomab"[Supplementary Concept] OR catumaxomab[tiab] OR antibody-drug*[tiab] OR monoclonal-antibod*[tiab] OR "Protein Kinase Inhibitors"[Mesh] OR "Angiogenesis Inhibitors"[Mesh] OR "Matrix Metalloproteinase Inhibitors"[Mesh] OR "Histone Deacetylase Inhibitors"[Mesh] OR "ErbB Receptors"[Mesh] OR HER2[tiab] OR erbB-2[tiab] OR erbB2[tiab] OR erbB-1[tiab] OR erbB1[tiab] OR epidermal-growth-factor-receptor*[tiab] OR EGFR[tiab] OR EGF-receptor*[tiab] OR "Receptors, Vascular Endothelial Growth Factor"[Mesh] OR VEGF[tiab] OR vascular-endothelial-growth-factor-receptor*[tiab] OR VEGF-A[tiab] OR VEGFA[tiab] OR VEGFR[tiab] OR VEGFR-2[tiab] OR VEGFR2[tiab] OR VEGFR1[tiab] OR VEGFR-1[tiab] OR tyrosine-kinase[tiab] OR RTK[tiab] OR TIE2[tiab] OR TIE-2[tiab] OR "Proto-Oncogene Proteins c-met"[Mesh] OR c-MET[tiab] OR "Hepatocyte Growth Factor"[Mesh] OR hepatocyte-growth-factor[tiab] OR HGF[tiab] OR mammalian-target-of-rapamycin[tiab] OR mTOR[tiab] OR "CLDN18 protein, human"[Supplementary Concept] OR claudin-18*[tiab] OR anti-claudin[tiab] OR matrix-metalloproteinase*[tiab] OR MMPs[tiab] OR MMP-9[tiab] OR MMP9[tiab] OR histone-deacetylase[tiab]) AND (("Randomized Controlled Trial"[Publication

Type] OR "Controlled Clinical Trial"[Publication Type] OR random*[tiab] OR trial[tiab] OR placebo[tiab] OR groups[tiab] OR RCT[tiab] OR CCT[tiab] OR NCT0*[tiab] OR NCT1*[tiab] OR NCT2*[tiab] OR NCT3*[tiab] OR NCT4*[tiab] OR NCT5*[tiab] OR NCT6*[tiab] OR NCT7*[tiab] OR NCT8*[tiab] OR NCT9*[tiab] OR phase-1[tiab] OR phase-I[tiab] OR phase-2[tiab] OR phase-II[tiab] OR phase-3[tiab] OR phase-III[tiab] OR placebo[tiab]) NOT ("Animals"[Mesh] NOT "Humans"[Mesh])) AND ("2015/01/01"[Date-Entry]: "2020/12/09"[Date-Entry]).

The review was conducted according to the PRISMA guidelines for systematic reviews [65].

3.4. Data Selection

Three reviewers (S.D., C.F., and L.P.) independently screened the titles and abstracts and identified the appropriate studies based on the selection criteria.

In addition, a fourth author (R.R.) reviewed the selected abstracts. Subsequently, authors obtained the full texts to verify their appropriateness.

Disagreements between reviewers were resolved by repeated examination of the original articles and discussions within the team.

3.5. Quality Assessment

The quality of the included studies was evaluated by two independent reviewers (S.D. and C.F.) with the application of the Cochrane risk-of-bias tool for randomized trials (*RoB 2*) [66].

The selection of reported results, measurement of outcomes, missing outcome data, and deviation from the intended interventions and randomization processes were assessed for each trial.

3.6. Statistical Analysis

R software (version 4.0.5, R Foundation for statistical computing, Vienna, Austria) was used for pooling data and statistical analysis. For time-to-event outcomes (OS, PFS) and for severe adverse effects, we combined data using the generic inverse variance method presenting measurements of treatment effects as hazard ratios (HRs) and 95% confidence intervals (CIs). As in the 2016 Cochrane review, as the design of the agents of interest is based on a different mechanism (targeting different pathways), we used a random-effects model for primary analyses. Tests for heterogeneity were conducted using the Chi^2 test. We adopted the I^2 statistic to estimate the total variation across studies due to heterogeneity [67]. If high levels of heterogeneity ($I^2 > 50\%$) for primary outcomes were found, we explored possible sources using subgroup analyses. We did not perform tests for subgroup differences owing to the limited number of trials involved in each molecular prognostic biomarker subgroup.

4. Results

4.1. Literature Searches

The literature review and trial selection are detailed in Figure 2, based on PRISMA guidelines [65]. We conducted the search on the main electronic databases (950 articles found in MEDLINE, 4051 in EMBASE, and 1211 in CENTRAL) from 1 January 2015 to 9 December 2020 in collaboration with "Biblioteca Federata di Medicina, Università degli studi di Torino". A total of 6212 papers were identified and subsequently deduplicated, resulting in 4634 included studies. After the first screening, 4497 studies were excluded because they did not meet inclusion criteria. An additional 114 articles were excluded because they were phase II trials or subgroup analysis-based studies. The remaining 23 articles were carefully analyzed, and 14 were removed. Reasons for exclusions are summarized in Figure 2. Subsequently, we excluded another article due to the inclusion of its data in the previously published Cochrane review [58]. Although one of the remaining eight trials was available only as an abstract, its detailed data and final findings were reported both in an

American Society of Clinical Oncology presentation and on the *ClinicalTrials.gov* website; therefore, this study was not excluded [68].

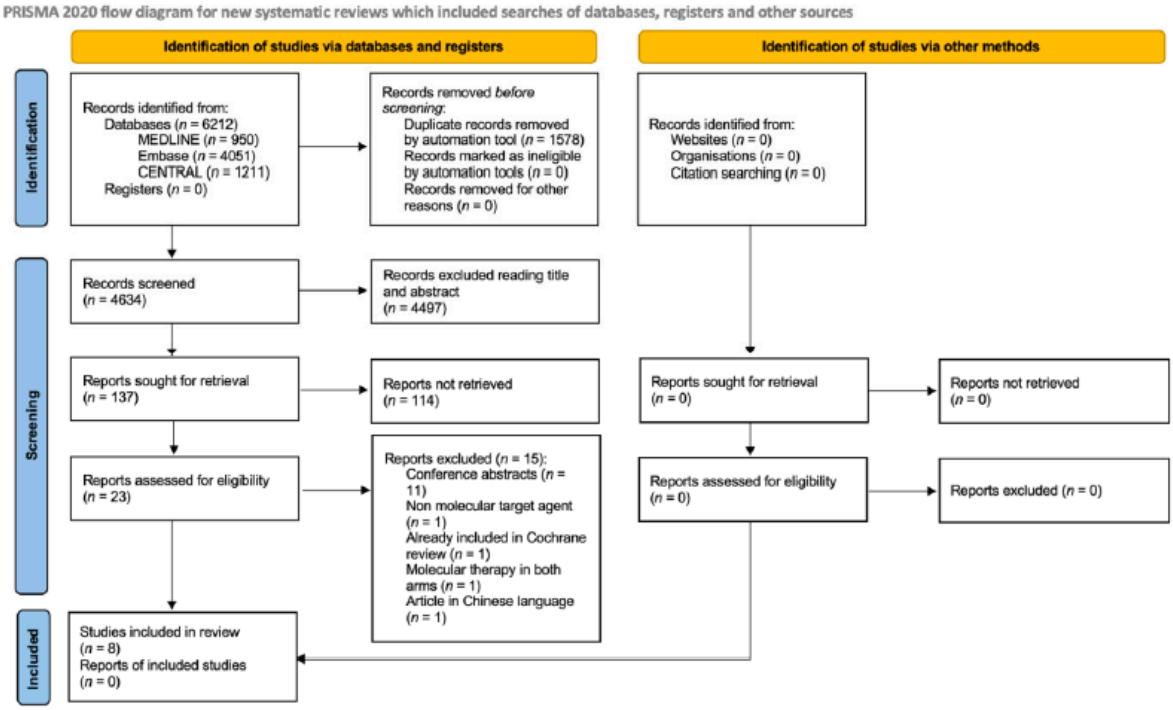


Figure 2. Prisma flow diagram.

Finally, 8 randomized controlled phase III trials with a total of 4223 enrolled patients were included in the present systematic review [32,68–74].

4.2. Risk of Bias in the Included Studies

The risk of bias in the included RCTs as calculated with the RoB2 tool is detailed in Figure 3.

The overall analysis resulted in half of the included trials showing a low risk of bias for all items [32,68,70,73], while some concerns were registered in one domain only in each of the remaining four studies [69,71,72,74].

4.2.1. Study Characteristics

The main features of the enrolled trials are detailed in Table 2. Overall, more than half of the patients (76%) did not receive any previous line of chemotherapy, 14.7% of them were given only one line, and 6.4% and 3% were provided with two and three lines, respectively, before being included in the RCTs. Most of the trials evaluated OS as the primary endpoint, whereas three studies analyzed PFS.

Table 2. Patient characteristics of the included studies (overall cohorts).

Author, Year, Acronym	Nr Total Pts (EXP/CTR)	Treatment Line (%)	Primary Endpoint	Setting	Molecular Target	EXP Arm	CTR Arm	Tumor Stage (%)	Tumor Site (%)	Notes
Li [69], 2016	267 (176/91)	III (65.1) IV(34.8)	OS, PFS	Adj	VEGFR-2	Apatinib	Placebo	III IV	Stomach (41.9) GEJ (13.5)	
Shah [74], 2017, METGastric	562 (279/283)	I (81.7) II (18.3)	OS	Adj	MET	Onartuzumab + FOLFOX6	Placebo + FOLFOX6	IV	Stomach (76.9) GEJ (23.1)	Enrollment was stopped early due to sponsor decision, which was agreed with the IDMC, due to a lack of efficacy in a phase II trial also assessing mFOLFOX6 plus onartuzumab
Thuss-Patience [32], 2017, GATSBY	345 (228/117)	II	OS	Adj	HER-2	Trastuzumab + Emtasine	Taxane	III (4) IV (95.9)	Stomach (68.1) GEJ (31.9)	
Catenacci [70], 2017RILOMET-1	609 (304/305)	I	OS	Adj	HGF	Rilotumumab + ECX	Placebo + ECX	IV (93.1) III (6.9)	Stomach (69.3) GEJ (20.4) Distal esophagus (10.3)	Study treatment was stopped early after a higher number of deaths in the rilotumab group.
Cunningham [71], 2017, UK Medical Research Council ST03	1063 (533/530)	I	OS	Periop	VEGF	Bevacizumab + ECX	ECX	Early (0.6) Advanced (91.7) Metastatic (0.19)	Stomach (55.7) GEJ (30.8) Distal esophagus (13.5)	EGJ type III was classified as gastric cancer
Fuchs [72], 2019, RAINFALL	645 (326/319)	I	PFS	Adj	VEGFR-2	Ramucirumab + Fluoropyrimidine + Cisplatin	Placebo + Fluoropyrimidine + cisplatin	IV (100)	Stomach (74.6) EGJ (25.3)	
Lorenzen [73], 2020, RADPAC	300(150/150)	II (57.7) III (31.7) IV (10.7)	OS	Adj	mTOR	Paclitaxel + Everolimus	Placebo + Paclitaxel	III IV	Stomach (41) GEJ (58.7)	
Shah [68], 2020 GAMMA-1	432 (218/214)	I	OS	Adj	MMP9	Andecaliximab + mFOLFOX6	Placebo + mFOLFOX6	IIIIV	Stomach (66) GEJ (34)	
Summary of Findings	4223 (2214/2009)	I (76) II (14.7) III (6.4) IV (3)	OS (87.5%) PFS (25%)	Adj 9 (87.5%) Periop 1 (12.5)					Stomach (63.5) EGJ (28.7) Esophagus (4.9)	2 trials were stopped early

Nr. number; pts: patients; Adj: adjuvant treatment; Periop: perioperative treatment; OS: overall survival; PFS: progression free survival; EXP: experimental; CTR: control; GEJ: gastroesophageal junction; FOLFOX6: fluorouracil leucovorin oxaliplatin; ECX: epirubicin, cisplatin, and capecitabine; mFOLFOX6: modified oxaliplatin, leucovorin, and fluorouracil; MMP9: matrix metalloproteinase 9.

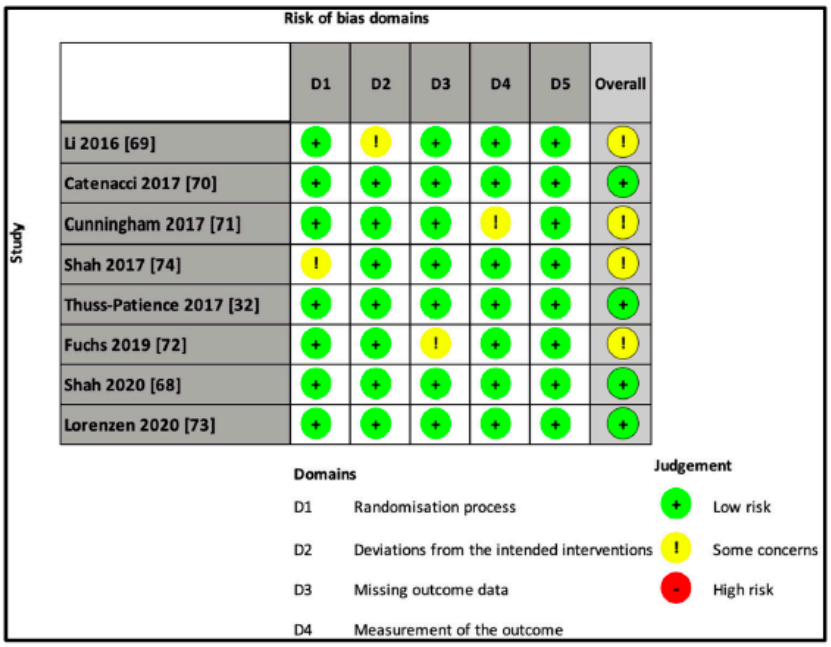


Figure 3. Risk of bias. To assess the risk of bias of each included study, the revised version of the Cochrane tool (RoB 2) was employed. The RoB 2 tool is structured into domains through which bias might be introduced into the result. These domains were identified based on both empirical evidence and theoretical considerations.

In contrast to the other RCTs, Cunningham et al. [71] designed a study in a perioperative setting, also enrolling patients in early stages. However, generally, the patients included in this systematic review mostly had locally advanced, recurrent, or metastatic malignancies.

All selected trials analyzed both gastric and EGJ cancers; moreover, two of these trials also enrolled patients with esophageal malignancies [70,71].

The studies evaluated heterogeneous types of MTs with different targets: three of them used VEGFR targeting agents (apatinib [69], bevacizumab [71], ramucirumab [72]), two trials focused on c-MET inhibiting agents (onartuzumab [74] and rilotumumab [70]), one administered trastuzumab plus emtasine (anti-HER2) [32], and the remaining two studies investigated everolimus (anti-mTOR) [73] and andecaliximab (anti-MMP9) [68].

The majority of RCTs analyzed the efficacy of MT in combination with conventional CT compared to conventional treatment alone, with or without placebo, while the GATSBY [32] study compared MT alone versus conventional therapy. Curiously, the study by Li et al. compared the efficacy and safety of MT alone with those of placebo alone [69].

Two RCTs were terminated prematurely due to negative results [70,74]. Notably, the RILOMET-1 study was halted due to a significantly higher number of deaths in the experimental arm than in the control arm during a planned interim safety analysis.

4.2.2. Survival Outcomes

All included RCTs analyzed both OS and PFS; results are detailed in Table 3. The median follow-up duration was available for seven of eight trials since the study by Li et al. [69] did not report follow-up information. It was 15.9 months (range, 6.2–39.1 months) for the experimental group and 15.2 months (range, 5.6–36.2 months) for the control group.

Table 3. Characteristics of studies included in meta-analysis, along with information on primary outcomes. The positive (green dot) or negative (red dot) outcomes of each study are reported, consistent with its primary endpoint.

				OS				PFS				Results
Author, Year, Acronym	**EXP**	**CTR**	**Nr**	**HR**	**Low**	**High**	***p* Value**	**HR**	**Low**	**High**	***p* Value**	
Li [69], 2016	Apatinib	Placebo	267 (176/91)	0.709	0.537	0.937	0.015	0.444	0.331	0.595	<0.001	🟢
Shah [74], 2017, METGastric	Onartuzumab + FOLFOX6	Placebo + FOLFOX6	562 (279/283)	0.82	0.59	1.15	0.24	0.90	0.71	1.16	0.43	🔴
Thuss-Patience [32], 2017, GATSBY	Trastuzumab + Emtasine	Taxane	345 (228/117)	1.15	0.87	1.51	0.86	1.13	0.89	1.43	0.31	🔴
Catenacci [70], 2017, RILOMET-1	Rilotumab + ECX	Placebo + ECX	609 (304/305)	1.34	1.10	1.63	0.003	1.26	1.04	1.51	0.016	🔴
Cunningham [71], 2017, UK Medical Research Council ST03	Bevacizumab + ECX	ECX	1063 (533/530)	1.08	0.91	1.29	0.36	1.05	0.89	1.23	0.56	🔴
Fuchs [72], 2019, RAINFALL	Ramucirumab + Fluoropy-rimidine + Cisplatin	Placebo + Fluoropy-rimidine + Cisplatin	645 (326/319)	0.962	0.801	1.156	0.68	0.753	0.607	0.935	0.011	🔴
Lorenzen [73], 2020, RADPAC	Paclitaxel + Everolimus	Placebo + Paclitaxel	300 (150/150)	0.93	0.73	1.18	0.544	0.88	0.70	1.11	0.273	🔴
Shah [68], 2020 GAMMA-1	Andecaliximab + mFOLFOX6	Placebo + mFOLFOX6	432 (218/214)	0.93	0.74	1.18	0.56	0.84	0.67	1.04	0.10	🔴

Nr: number; HR: hazard ratio; OS: overall survival; PFS: progression free survival; EXP: experimental; CTR: control; XELOX: capecitabine and oxaliplatin; FOLFOX6: fluorouracil leucovorin oxaliplatin; ECX: epirubicin, cisplatin, and capecitabine; mFOLFOX6: modified oxaliplatin, leucovorin, and fluorouracil; MMP9: matrix metalloproteinase 9. 🟢 positive study. 🔴 negative study.

The meta-analysis showed that the global OS after targeted therapy was comparable to that after conventional therapy, with an HR of 0.99 (95%CI: 0.84; 1.16; $p = 0.867$, $I^2 = 62\%$) (Figure 4).

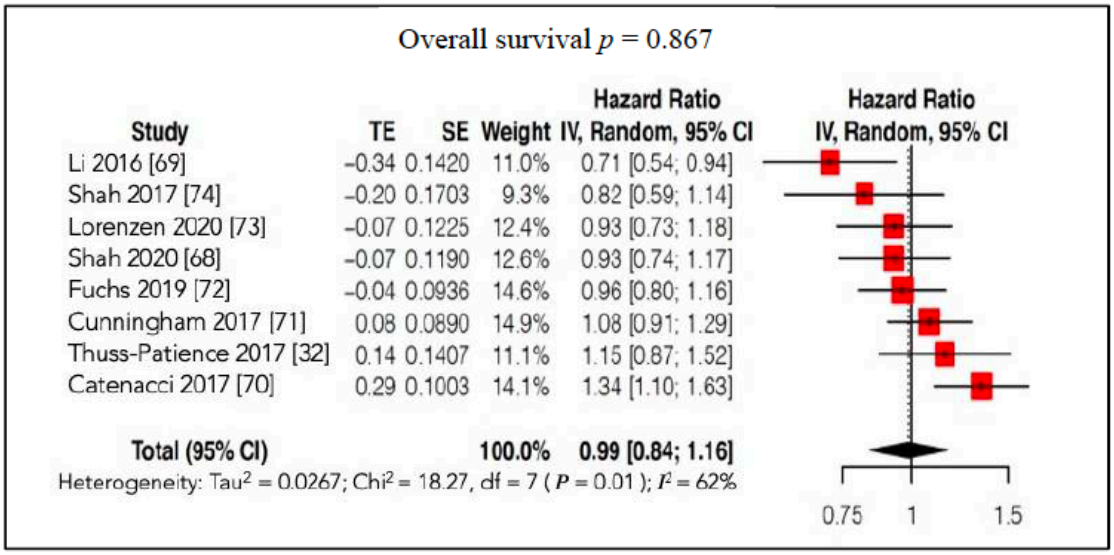


Figure 4. Forest plot of comparison: molecular-targeted therapy alone/plus chemotherapy versus chemotherapy alone/placebo. Main analyses; outcome: overall survival.

Subsequently, OS was assessed considering 2 MT subgroups (Figure S1) according to the main categories of TKR inhibitors (VEGFR or c-MET inhibitors) administered to patients. This analysis confirmed the absence of a significant difference in survival between patients treated with a particular type of MT and those treated with conventional CT or placebo. In a total of 2942 patients, a meta-analysis of PFS was carried out using individual patient-level trial data. Similar to the OS findings, the use of MT did not show any improvement in PFS compared to conventional therapy or even to no treatment (HR 0.88, 95%CI: 0.68; 1.14, $p = 0.286$, $I^2 = 84\%$) (Figure 5).

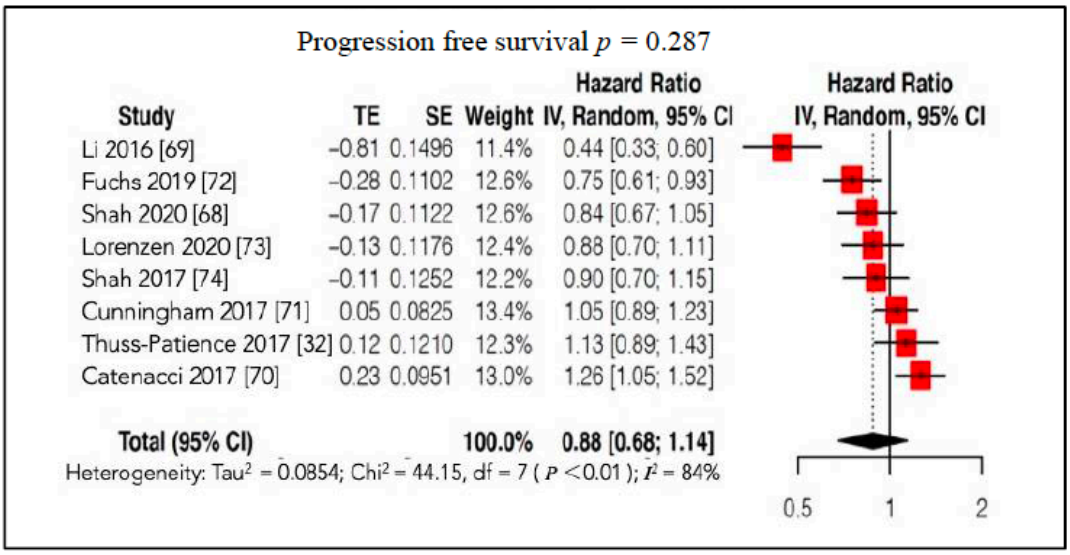


Figure 5. Forest plot of comparison: molecular-targeted therapy alone/plus chemotherapy versus chemotherapy alone/placebo. Main analyses; outcome: progression free survival.

Furthermore, the MT subgroup analysis (inhibitors of VEGFR vs. inhibitors of c-MET) confirmed the findings of the overall analysis (Figure S2).

4.2.3. Secondary Outcomes

Overall Response Rate

Seven of the eight studies reported data about the ORR based on RECIST criteria (Table 4). The majority of these trials did not find a significant difference in ORR between the experimental and control groups. The RILOMET-1 study reported even a significantly better ORR in the control group [70], while the recent GAMMA-1 study registered a slightly higher ORR in the experimental arm ($p = 0.049$) [68].

Quality of Life

Only two RCTs evaluated patients' QoL with the application of the EORTC QLQ-C30 global health status scale [69,72]. The QLQ-C30 response rate was high in every questionnaire domain in both studies, without any significant difference between the two groups.

Table 4. Overall response rate and quality of life. This table summarizes the overall response rate based on RECIST criteria reported in the experimental and in the control arm for each study. The quality of life was reported according to EORTC QLQ-C30 questionnaire, which measure cancer patients' physical, psychological, and social functions. This questionnaire is composed of multi-item scales and single items.

Author, Year, Acronym	Overall Response Rate			Quality of Life EORTC QLQ-C30
	EXP arm (%)	CTR arm (%)	*p* value	
Li [69], 2016	2.84	0.0	0.1695	No differences ($p > 0.05$)
Shah [74], 2017, METGastric	40.6	46.1	0.25	nd
Thuss-Patience [32], 2017, GATSBY	20.6	19.6	0.8406	nd
Catenacci [70], 2017, RILOMET-1	29.8	44.6	0.0005	nd
Cunningham [71], 2017, UK Medical Research Council ST03	41	42	0.70	nd
Fuchs [72], 2019, RAINFALL	41.1	36.4	0.17	HR 1.029 (0.786, 1.347)
Lorenzen [73], 2020, RADPAC	8	7.3	nd	nd
Shah [68], 2020, GAMMA-1	50.5	41.1	0.049	nd

EXP: Experimental; CTR: Control.

Serious Adverse Effects

Finally, we proceeded to analyze the safety of the experimental arm compared to that of the control arm in terms of emergent SAE (grade ≥ 3) and SAE-related deaths. All of the articles described the occurrence of SAE. However, the meta-analysis of the available data showed that MT did not increase the number of SAEs compared with conventional treatment (HR 0.96, 95%CI: 0.78; 1.19, $I^2 = 23\%$) (Figure 6).

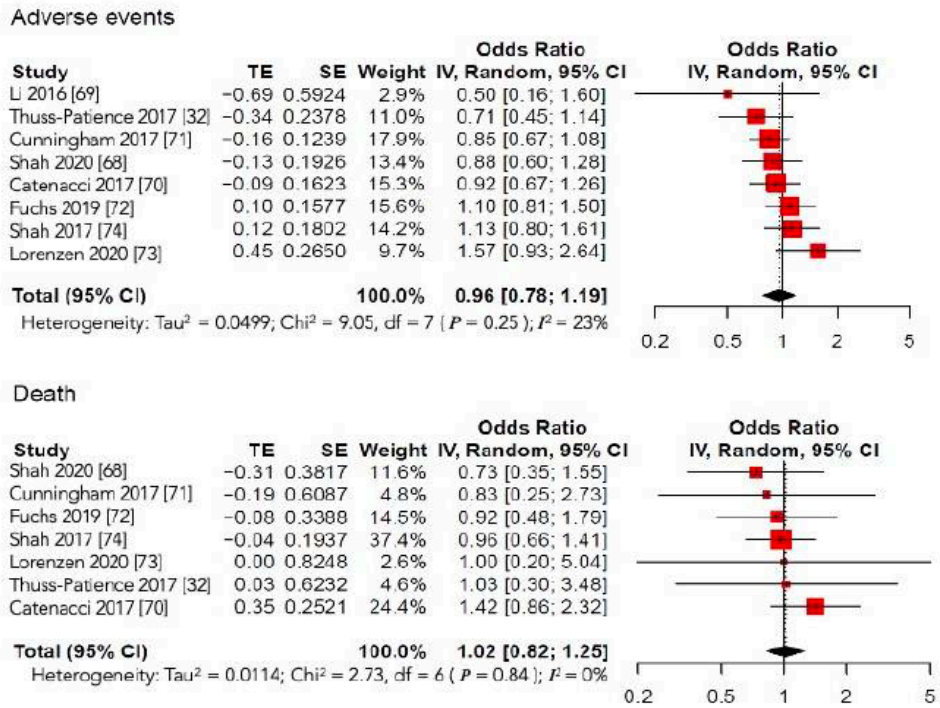


Figure 6. Forest plots of comparison: molecular-targeted therapy alone/plus chemotherapy versus chemotherapy alone/placebo. Secondary analyses; outcome: serious adverse effects and related-deaths.

The number of adverse events with fatal outcomes was detailed in seven of the eight included studies, as the trial by Li et al. [69] did not mention these data. As with the incidence of SAE, the administration of MT with or without conventional CT did not increase the rate of treatment-related deaths (HR 1.02, 95%CI: 0.82; 1.25, I^2 = 0%) (Figure 6). Only the RCT by Catenacci et al. [70], investigating the safety and efficacy of rilotumumab (anti-cMET agent), was prematurely stopped due to a higher proportion of fatal adverse events, mostly due to disease progression, in the experimental arm than in the control arm. We used the fixed-effect model according to the absence of significant heterogeneity in both meta-analyses.

5. Discussion

GC is still characterized by a poor prognosis, particularly in cases of metastatic or recurrent disease and in locally advanced stages. The identification and introduction of effective and safe molecular therapies in clinical practice lag behind other malignancies, such as lung and breast cancers. To the best of our knowledge, this is the most recent systematic review and meta-analysis of emergent targeted therapies for GC.

Unfortunately, our findings showed that molecular therapies do not provide a clear survival benefit compared to conventional CT in the case of advanced or metastatic GC.

In 2016, the Cochrane group published the largest systematic review and meta-analysis investigating the survival benefit of MTs for GC patients, with or without conventional treatment. The Cochrane authors identified 11 RCTs (phase II and III studies), and the conclusion was "Adding molecular-targeted treatment to chemotherapy may have a small effect on survival and on stopping further development of the disease, compared with chemotherapy alone, but the evidence is of low quality".

In the past five years, only eight new phase III RCTs have been conducted.

Most of these studies failed to demonstrate the superiority of MT with or without conventional CT compared with conventional treatment alone or with placebo in terms of survival outcomes. Moreover, two of these eight trials were terminated prematurely. The METGastric Phase III trial was stopped early because of negative results reported in a concomitant Phase II study that concluded: "The addition of onartuzumab to mFOLFOX6 in gastric cancer did not improve efficacy in an unselected population or in a MET immunohistochemistry-positive population" [74,75]. The RILOMET-1 was interrupted prematurely because a safety control committee found more deaths in the experimental arm than in the control arm during a planned interim analysis of safety and survival outcomes [70].

The RCT published by Li was the only positive study; it reported a clear survival benefit in patients with GC treated with apatinib (a VEGFR2 inhibitor) compared with those receiving a placebo in terms of both OS (7.6 vs. 5.0 months, $p = 0.0027$) and PFS (2.8 vs. 1.9 months, $p < 0.001$), with an acceptable SAE rate [69]. Accordingly, in 2014, the China Food and Drug Administration approved the use of apatinib as a third-line treatment for metastatic GC.

Despite this positive report, the overall meta-analysis did not show any significant differences in OS and PFS between the experimental (MT) and control arms.

Furthermore, the subgroup analysis according to the type of MT administered (VEGFR or c-MET inhibitors) failed to show a significant prolongation of OS and/or PFS in the experimental arm. Notably, our results may have been unable to identify significant differences between the two arms due to the high heterogeneity found among the included studies. On account of this statistical bias, we conducted two further meta-analyses matching our OS and PFS findings with those reported in the Cochrane review [76–81] (Figure 7a,b; Table S1). Regrettably, these new cumulative analyses maintained high heterogeneity and could not document any survival advantage when MT was added to conventional treatment or administered alone compared to conventional CT or to a placebo.

Most of the included trials reported no differences in the ORR evaluated according to RECIST criteria [63] between the two treatment arms, with the exception of the RILOMET-1

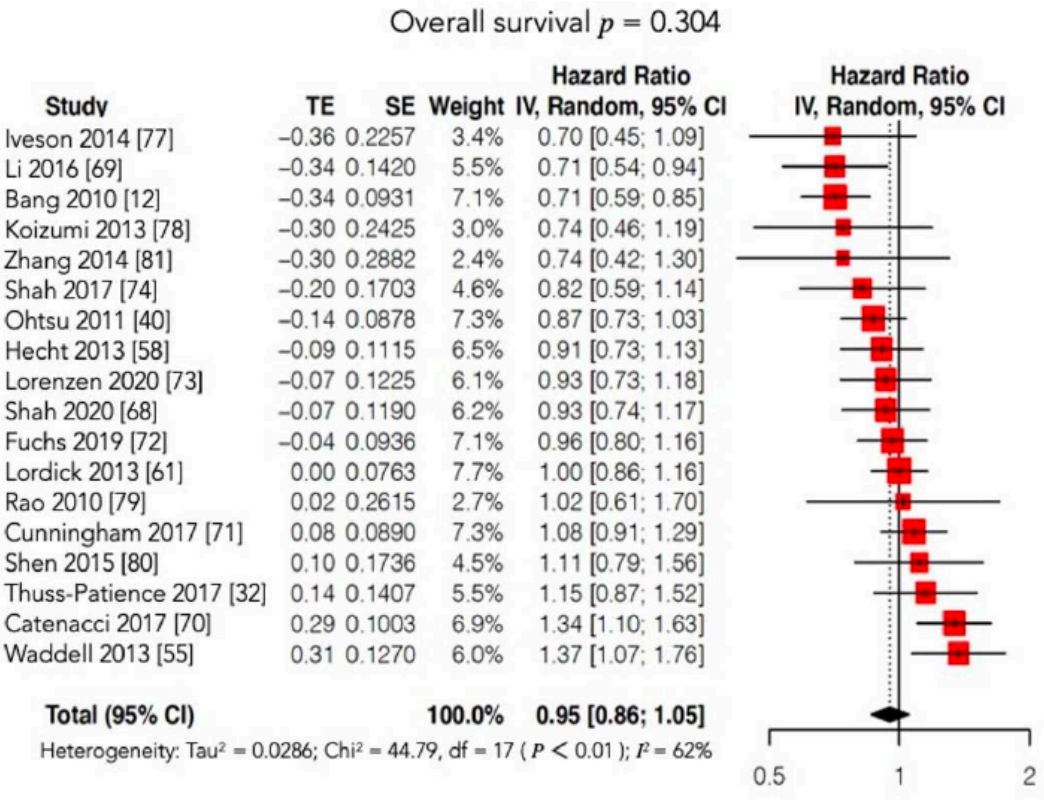
study [70], which registered a significantly worse response in the experimental arm, and in the GAMMA-1 trial [68], which, on the contrary, reported a significantly better result in the MT group.

Quality of life was mentioned only in the study by Li et al. [69] and in the RAINFALL study [72] without any significant differences between the two groups.

Finally, the number of serious adverse effects and SAE-related deaths did not increase in the experimental arm. Additionally, the analysis of secondary outcomes confirmed that, to date, the supposed advantage of the administration of MT vs. conventional CT alone is unclear.

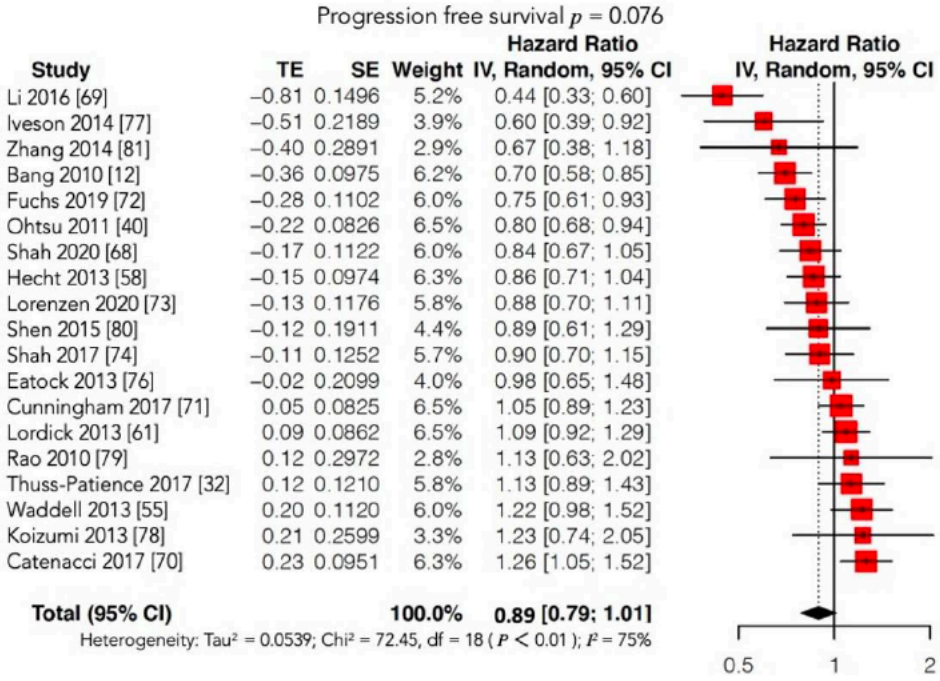
In addition, most of the investigated targeted therapies available to date are very expensive; therefore, it is mandatory to evaluate the cost-effectiveness as well. In 2017, Chen et al. [82] evaluated the relationship between the efficacy and the costs of apatinib as a third-line treatment in metastatic GC and concluded that this type of treatment is not cost-effective at all, while another author stated that apatinib is likely to be cost-effective only for patients with solid insurance [83]. Other authors analyzed the cost-effectiveness ratio of ramucirumab + paclitaxel as a second line treatment in AGC as proposed by Wilke et al. [47], concluding that this regimen was cost-ineffective and suggesting that its indirect charges to society be considered [84,85].

Finally, although three MTs have been approved by the FDA (trastuzumab, trastuzumab–deruxtecan, and ramucirumab) and a fourth one by the China Food and Drug Administration (apatinib), most phase III RCTs assessing novel molecular agents failed to demonstrate a survival advantage over conventional treatments. Consistent with the literature, we found four possible reasons for these negative results.



(a)

Figure 7. *Cont.*



(b)

Figure 7. **(a)** Forest plot of comparison: molecular-targeted therapy alone/plus chemotherapy versus chemotherapy alone/placebo. Main analyses; outcome: overall survival (data from Cochrane and present review pooled). **(b)** Forest plot of comparison: molecular-targeted therapy alone/plus chemotherapy versus chemotherapy alone/placebo. Main analyses; outcome: progression free survival (data from Cochrane and present review pooled).

First, only in recent times has GC undergone wide investigational programs from a molecular perspective, which has highlighted the importance of patient selection because of the high number of molecular mutations found in GC [86]. Indeed, several molecular alterations characterizing GC subtypes have been identified and analyzed in the past decade, as in the case of CIN tumors, which manifest the most frequent TKR amplifications, and in the case of 80% of EBV tumors, which display *PIK3CA* mutations [87].

Second, GC is often characterized by a high grade of heterogeneity, both inside the primary tumor and in distant metastases. Several studies clearly demonstrated the intratumoral heterogeneous pattern of HER2 and c-MET expression [88,89]. Some authors have suggested inactivating alterations to the phylogenetic tree trunk because they promote cancer growth and are present in every tumor cell [90]. Unfortunately, no trunk mutations have been discovered in GC.

Third, several preclinical trials have recently documented a strict relationship between c-MET amplification and copy number and the response grade to anti-MET therapies [91,92] and that c-MET expression alterations are found in only 2% of GCs. However, in clinical trials investigating anti-MET agents, no patient selection was done. This could be one of the reasons for RILOMET-1 and METGastric trial failure.

Finally, many studies have shown different escape mechanisms of cancer cells that could shorten the duration of or even nullify the response to targeted therapies [93,94]. For example, c-MET-addicted GC could overcome c-MET blockade through HER family receptor expression activation. Recently, Apicella et al. showed that combined molecular

therapy with anti-MET/EGFR leads to a complete and durable response [91]. For this reason, PDX and PDOX are valuable preclinical tools in validating new targeted therapies tailored to patients' cancer molecular expression [14,15,95].

6. Conclusions

The results of this systematic review and meta-analysis showed that despite their newly documented safety, the molecular therapies available to date for advanced and metastatic gastric cancer do not present clear survival benefits. These unfavorable results are mostly related to inadequate patient selection. Targeted therapies are promising treatments for patients with locally advanced, metastatic, or recurrent gastric cancer as they are for other types of tumors. However, their clinical validation requires accurate patient selection, particularly related to driver oncogene amplification and copy number, and it should take into account preclinical models investigating cancer heterogeneity and escape mechanisms.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cancers13164094/s1, Figure S1. Overall survival of molecular therapies subgroups, Figure S2. Progression free survival of molecular therapies subgroups, Table S1. Cochrane studies' characteristics.

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Abbreviations

GC: gastric cancer, AGC: advanced gastric cancer, CT: chemotherapy, EBV: Epstein–Barr virus, MSI: microsatellite instable, CIN: chromosomal instable, GS: genomically stable, MT: molecular therapy, FDA: Food and Drug Administration, fAGC: far advanced gastric cancer, PDX: patient-derived xenograft, PDOX: patient-derived orthotopic xenografts, RCT: randomized controlled trial, EGFR: epidermal growth factor receptor, TKR: tyrosine kinase receptor, OS: overall survival, DFS: disease free survival, PFS: progression free survival, DM1: emtansine, ADC: antibody-drug conjugate, VEGF: vascular endothelial growth factor, ALK: anaplastic lymphoma kinase, NSCLC: non-small cell lung cancer, mTOR: mammalian target of rapamycin, cMET: hepatocyte growth factor receptor, HGFR: hepatocyte growth factor receptor, HR: hazard ratio, CI: confidence interval, Chi^2: Chi-squared, I^2: I-square, ORR: overall response rate, QoL: quality of life, SAE: serious adverse effects, EGJ: esophagogastric junction.

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5. DISCUSSION

In these last decades, the oncologic approach has rapidly shifted from a phenotype-based empirical treatment to a more personalized and patient-centered management, based on tumor genetic profile.

This innovative strategy has led to significant results in neoplasms such as lung, breast, and colorectal carcinomas, where driver genes to which tumor cells are addicted have been identified. Unfortunately, the GC genetic landscape has been explored only very recently and only three molecular therapies are actually approved by the FDA for the treatment of GC (Trastuzumab, Ramucirumab and trastuzumab deruxtecan) (57,99,116). This is because most of phase III RCT studies failed to demonstrate the superiority of MT, with or without conventional CT, compared with conventional treatment alone or with placebo in terms of survival outcomes.

Hence, there is an urgent need for further studies able to identify targetable drivers in this tumor. To deeply explore the molecular mechanisms sustaining tumor growth and response to therapy, animal models are very useful. To date, PDXs are the best preclinical model to validate targets and positive/negative predictors of response to therapy (117,118). Indeed, this strategy combines the flexibility of preclinical analyses with the informative value of population-based studies.
We thus generated a molecularly annotated platform of gastro esophageal PDXs (the widest developed in an academic institution) by subcutaneous transplantation in NOD SCID mice, with the aim to identify and validate targets and optimize molecular treatments in GC. PDXs were generated by placing a sample of GC in a surgical performed subcutaneous pouch in mice's leg. Tumor growth was followed weekly with a caliber measurement. The overall engraftment rate of our PDXs (42%) was in line with that described by other authors (119). However, we observed that this rate could be sensibly affected by some characteristics of the tumor. Indeed, the histology (intestinal vs. diffuse), the stage (advanced vs. early), the presence of alterations in RTK/KRAS genes and the MSS status (MSI vs. MSS) significantly increased tumor engraftment, which correlated with tumor aggressiveness.

This platform also includes primary cell lines and 3D-coltured organoids. The availability of a comprehensive PDX platform allows to perform trials on a huge number of models sharing the same genetic mutation.

The preclinical value of such a platform relies on some requirements: first, it must include the whole spectrum of the described histotypes; second, the PDXs must recapitulate the histopathologic,

biologic, and genetic features of donor tumors. Our gastro-esophageal PDXs platform entails all Lauren's histotypes, including the diffuse subtype, unlike other PDX platforms which did not obtain PDXs from tumors of this subtype (119).

In our study, gene expression profile was well conserved among the original tumors, PDXs, and the *in vitro*–derived material. Overall, PDX models retained the principal characteristics of donor tumors.

Moreover, all molecular types according to TCGA classification and all the most frequent gastric cancer–based genomic alterations identified in public consortia were well represented in our platform. Hence, these results demonstrate that the platform captures the heterogeneity of human gastric tumors.

The major problem of PDXs is that transplanted tumors are located in an abnormal microenvironment and most of them are encircled by a pseudocapsule. For these reasons subcutaneous implantation very rarely allows metastatic dissemination, whereas the orthotopic implantation of human tumour in mice (PDOX) better mimics the original microenvironment of the tumour itself, increasing its metastatic capability. Regrettably, our review of the literature documented the lack of validated and feasible GC PDOX models. We created a modified model validity tool to provide an 'ideal set of validation criteria' for PDOX animal models that could be adjusted and applied to other models or studies.

In the meantime, we developed two novel techniques (tumor tracing and its implantation in submucosal coat) that allowed us to generate and validate a GC PDOX model that mimics the original microenvironment of the tumor, and which can be monitored *in vivo*. Indeed, we generated a tumor cell line producing green fluorescent protein (GFP) and luciferase by cell infection with the virus pRRLsIn.PPT.CMV.Luciferase.IresEMCwt.eGFP.pre. From these 2D cells we developed organoids, by seeding cells in a Matrigel dome. Organoids were trypsinized with Tryple 1x to avoid damage and injected subcutaneously in the mice. The cancer growth was checked by IVIS system one month later. This tumor was then additionally implanted in several other mice to create a PDX platform with the same GC line. Hence, one piece of this tumor was implanted into the submucosal layer of several mice's stomach to develop a PDOX platform. PDOX tumor engraftment and appearance of metastases were monitored using IVIS technology in vivo. Pathologic analysis was performed after animal sacrifice. This new promising model of GC PDOX could be useful to text new MTs for gastric cancer.

Based on this comprehensive PDX/PDOX platform we could identify a MSI/MSS signature that could help identify patients with different prognosis in the two groups. Despite their better clinical prognosis, MSI GCs were documented harbouring a more aggressive behavior in mice, testified by a

2-fold higher engraftment rate as compared with MSS tumors. This controversy is probably due to the MSI tumors' high mutational burden that stimulates the activation of the human immune system resulting in a decreased aggressiveness of the cancer. Otherwise MSI tumors could unleash their full malignancy in PDX and PDOX because of their immunodeficiency.

We performed a transcriptomic analysis on our PDX cohort that allowed the identification of a cancer cell–intrinsic MSI signature. Hence, we generated a MSI score to detect patients with lower recurrence rate; remarkably, our score was also able to identify some patients bearing MSS tumors endowed with MSI transcriptional traits who displayed better prognosis. This finding could lead to the selection of patients lacking the genetic MSI characteristics but displaying an MSI-like signature who could benefit from the treatment with Immuno or other PARP-type drugs.

As a second important finding derived from platform data analysis, we could document the presence of selected concurring driver alterations in EGFR-amplified tumors. The simultaneous amplification of MET/HER2/KRAS and co-mutations of KRAS/PIK3CA/PTEN were demonstrated in 53% and 35% of patients in the EGFR-amplified subgroups. We also identified mTOR pathway activation as a novel mechanism of resistance to EGFR targeted therapy and observed that it could be overcome by the combination of EGFR/mTOR inhibitors. This result points out that only a subgroup of patients with EGFR-amplified may significantly benefit from single agent anti-EGFR therapy. Interestingly, the preclinical trials we performed on EGFR-amplified PDX models showed that the combination of an EGFR MAb with an EGFR TKI was more effective than each monotherapy and resulted in a deeper and durable response (120,121). These observations have important clinical implications showing that the optimization of the therapeutic efficacy can be achieved with a dual EGFR inhibition, and the identification of potential mechanisms of primary resistance could be useful for further experimentally driven clinical trials. The association of lapatinib and cetuximab has already proven safe in a phase I trial (122); furthermore second-generation antibodies mixtures against high-affinity EGFR ligands, such as Sym004 and MM-151 (123,124), are into clinical evaluation, and the TORC signaling pathway is targetable with commercially available drugs.

Finally, through comparing the efficacy of trastuzumab monotherapy versus a dual therapy (trastuzumab + pertuzumab or lapatinib) in the subpopulation of HER2-positive cancers, we documented that the response grade was significantly higher with the combos, leading to durable responses which in the evaluated cases did not relapse even after drug withdrawal.

Our results are in line with literature data. Indeed several studies on HER2 "hyper"-amplified gastroesophageal cancers evidenced that the dual-HER2 blockade (trastuzumab plus either pertuzumab or lapatinib) led to complete and durable responses in more than half of models (62.5%) (125–127). We also showed that in resistant PDXs harbouring KRAS amplification, the recently FDA

approved antibody–drug conjugate trastuzumab-deruxtecan overcame KRAS-mediated resistance. Indeed, the co-occurrence of HER2 amplification and KRAS genomic alterations has been observed in 5% of GC patients in the TCGA database. Overall our results suggest that the role of dual-HER2 blockade strategies should be re-assessed by novel RCTs aimed at focusing the enrolment of patients with HER2-positive GC to those with “hyper”-amplified status.

6. CONCLUSIONS

Targeted therapies are promising treatments for patients with locally advanced, metastatic, or recurrent gastric cancer as they are for other types of tumors. However, their clinical validation requires accurate patient selection, particularly related to driver oncogene amplification and copy number, and taking into account preclinical models investigating cancer heterogeneity and escape mechanisms. Preclinical trials can benefit from the use of animal models (PDX and PDOX) that represent valuable tools to validate molecular targets and positive/negative predictors of response to therapy. Novel studies on gastric cancer engrafted models should strictly employ model validity tools and larger samples with possibly orthotopic implantation sites, mirroring as much as possible the donor tumour characteristics and microenvironment.

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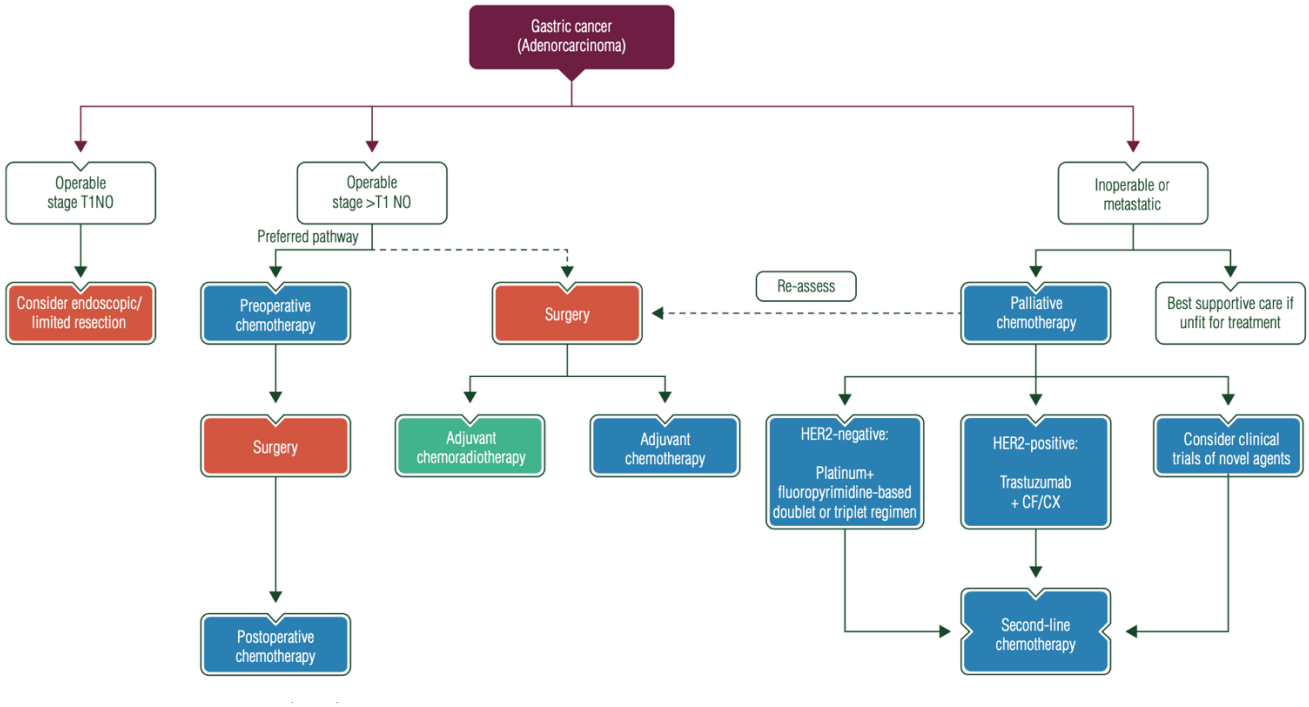
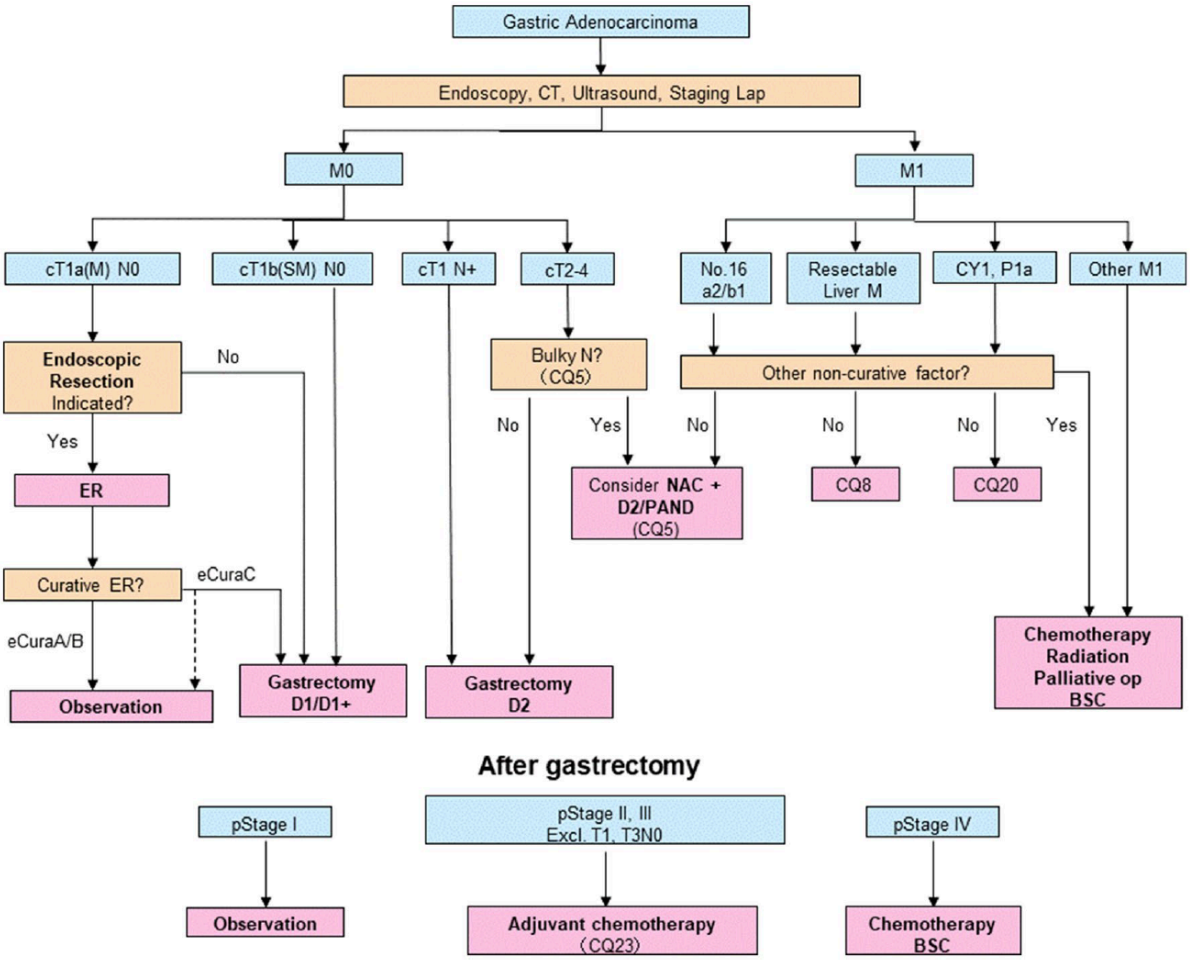
8. APPENDIX

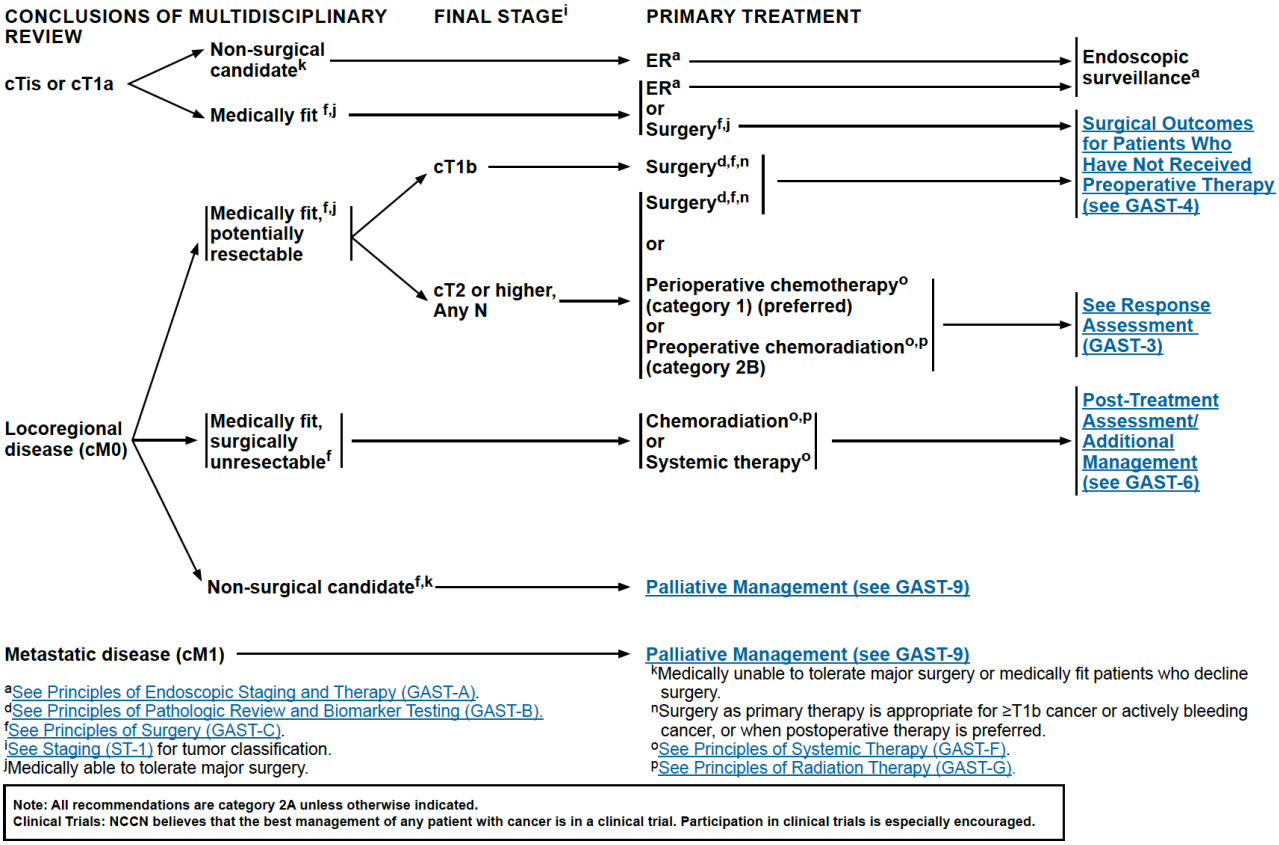
Algorithm of standard treatments.



a. European Society of Medical Oncology guideline 2016.



b. Japanese Gastric Cancer Association Guideline 2018 (5th Edithion).



c. National Comprehensive Cancer Network Guideline 2020.