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ORIGINAL ARTICLE

Personalized therapeutic strategies in HER2-driven gastric cancer

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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

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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.

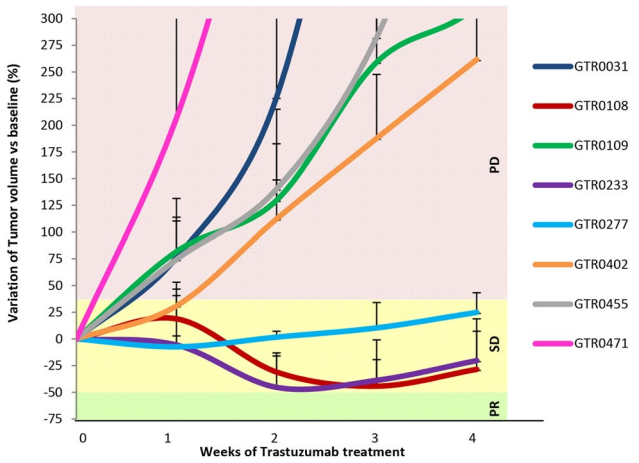


Fig. 1 Response to trastuzumab treatment in PDXs bearing high *HER2* CNG. The Spaghetti plot illustrates the effect of trastuzumab treatment (30 mg/kg) on PDXs with a *HER2* CNG ≥ 8 copies. Individual lines represent, for each PDX model, the mean percentage variation in tumor burden, from treatment start (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; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$.

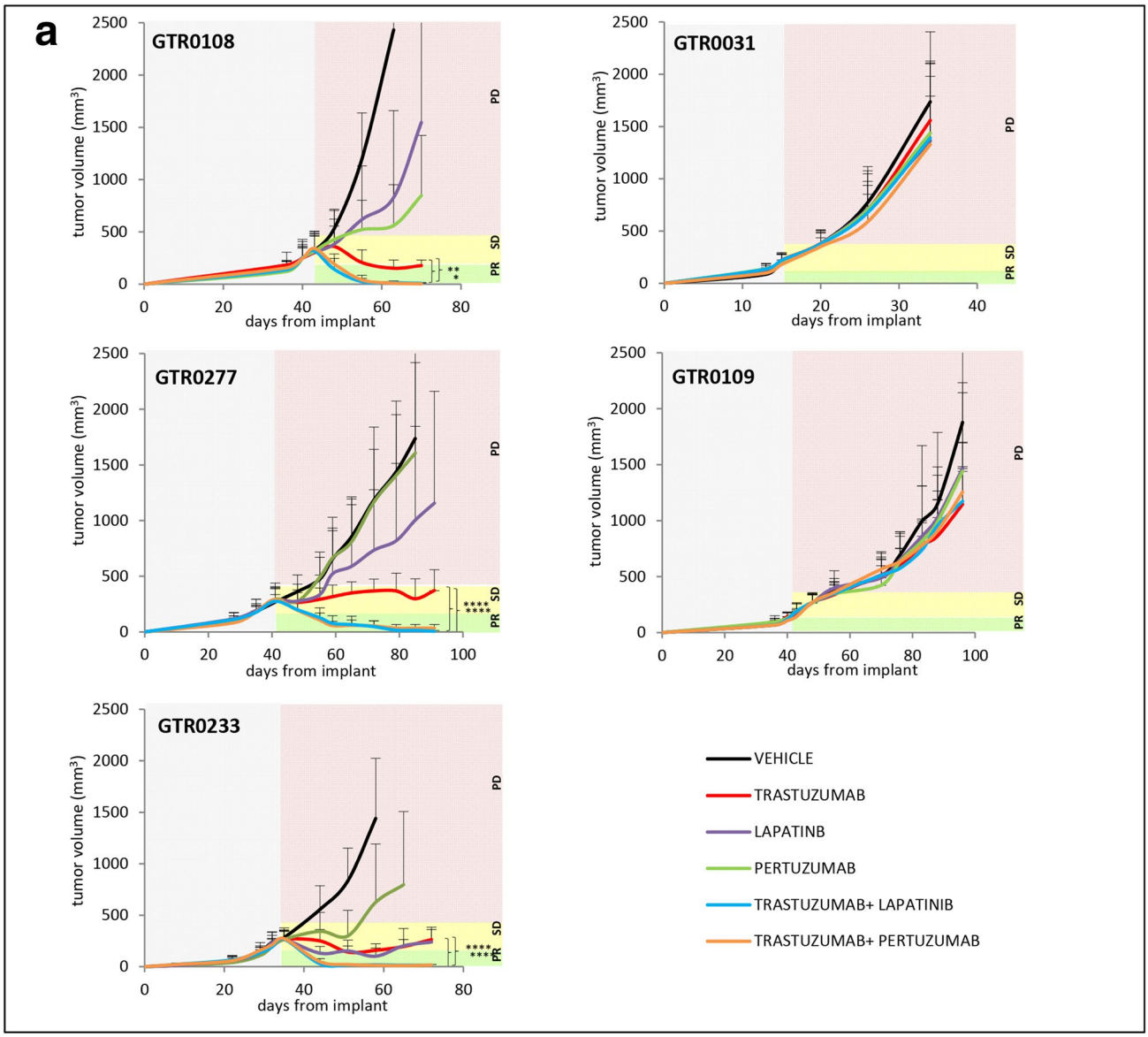
Genomic sequencing

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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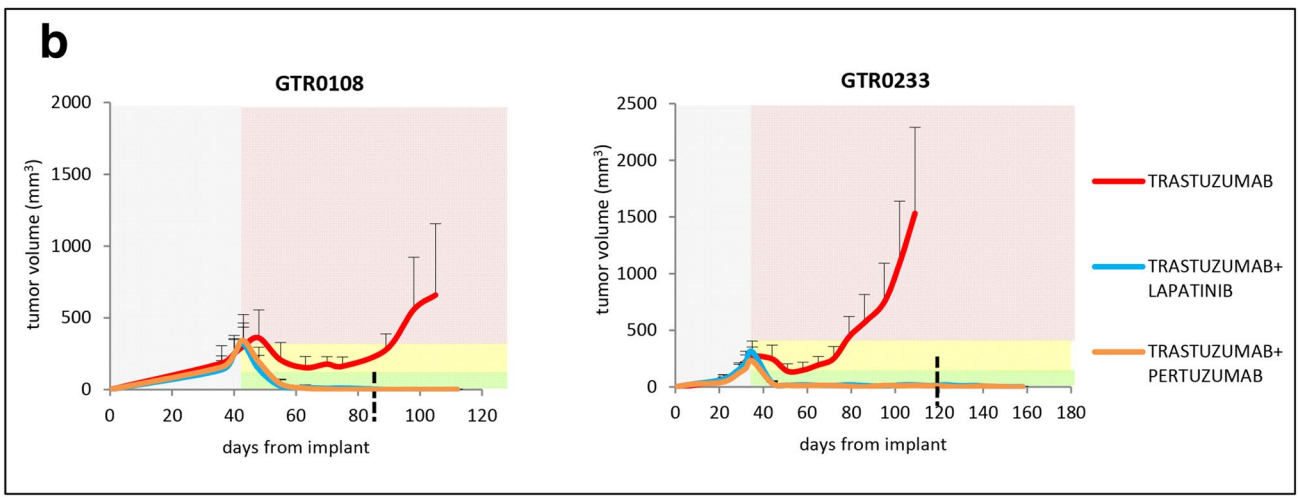
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◂**Fig. 2** Dual HER2 blockade is the most effective and durable treatment in *HER2*-amplified PDXs. **a** Tumor growth curves of mice cohorts derived from GTR0108, GTR0233, GTR0277, GTR0031 and GTR0109 patients, treated with the HER2 inhibitors trastuzumab, 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

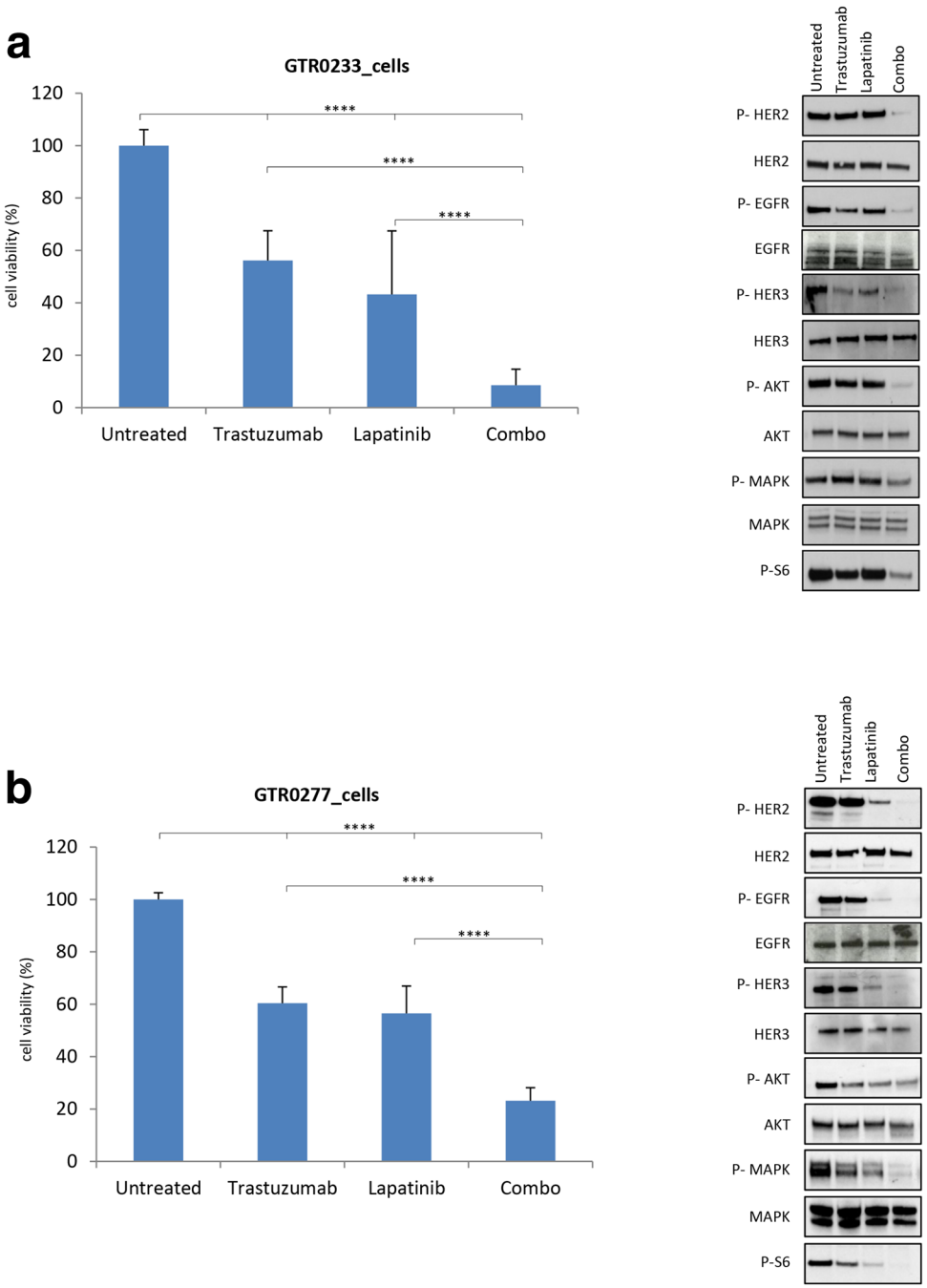


Fig. 3 Dual HER2 blockade is more effective than trastuzumab alone in GTR0233 and GTR0277 PDX-derived cells in vitro. Cell viability assay performed on GTR0233 (**a**, left panel) and GTR0277 (**b**, left panel) tumor-derived cells, upon treatment for 6 days with the indicated drugs at IC50 for each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

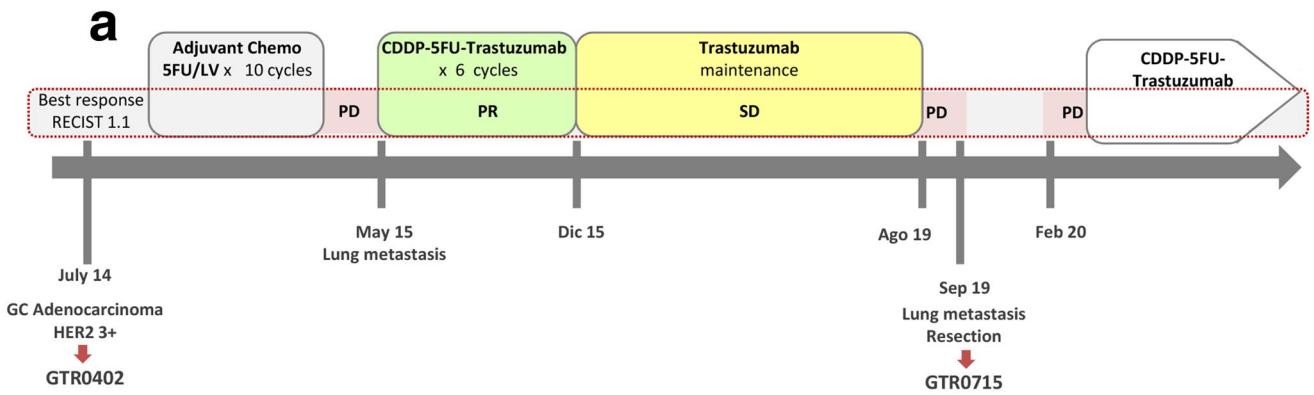
Intriguingly, we noticed that the two PDX models presenting 8–10 *HER2* copies (namely GTR0109 and GTR0031) did not show response to trastuzumab and did not get any benefit from the combo treatment. We thus performed an additional trial on another available model, GTR0471, displaying the same range of *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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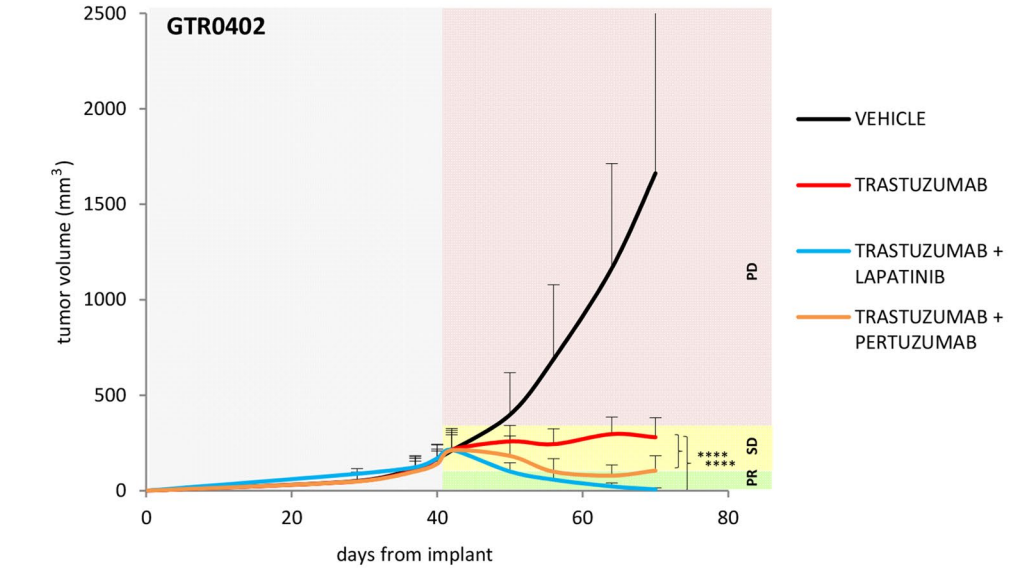
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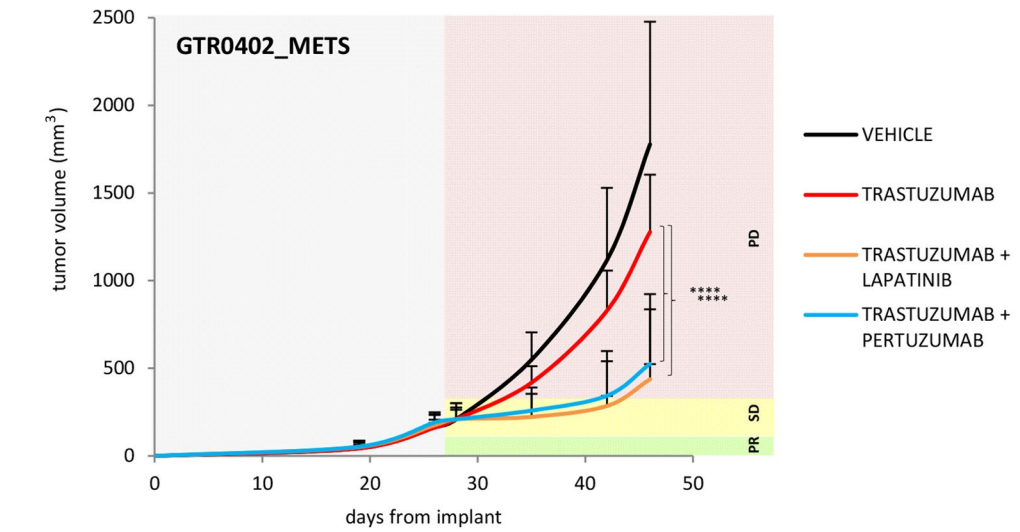
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◂**Fig. 4** PDX models recapitulate patients' response to trastuzumab. **a** Summarized clinical course of the GTR0402 patient. Grey-lined boxes indicate periods of administration of the indicated therapeutic agents. Grey vertical lines indicate timing 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.

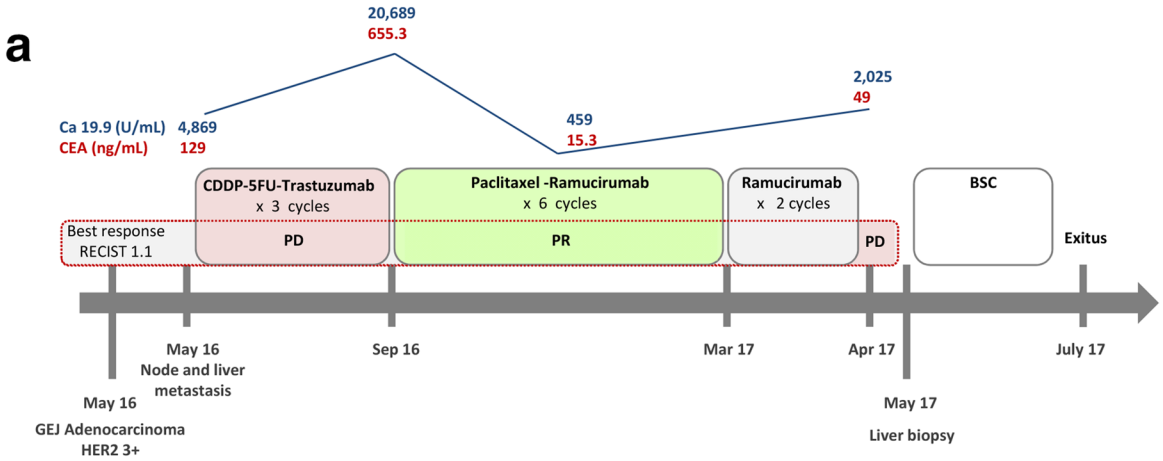
A non-cell autonomous mechanism sustains adaptive secondary resistance to HER2 inhibition

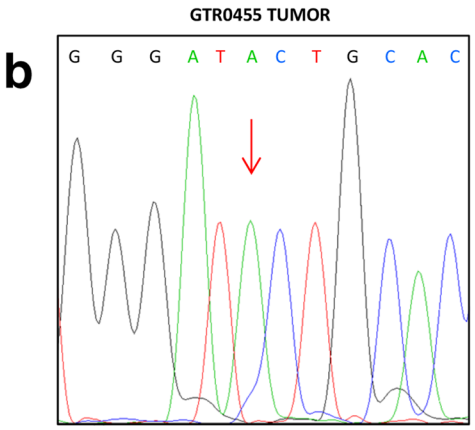
As already shown (Fig. 2), prolonged treatment of the GTR0233 PDX with anti-HER2 compounds in monotherapy resulted in tumor relapse (Figs. 2b, 6a). The genomic analysis of resistant tumors did not show any putative genomic alterations likely sustaining resistance to HER2 inhibition (data not shown). 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.

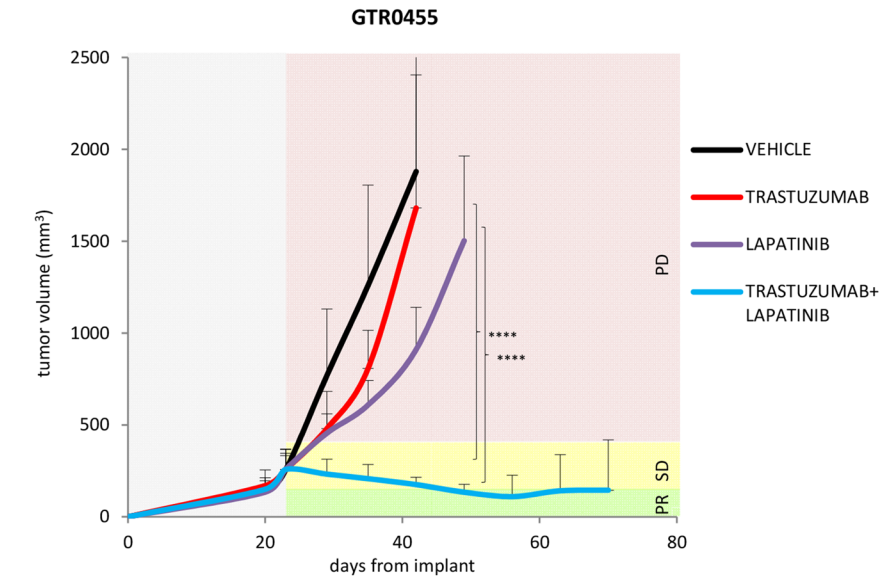
Discussion

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

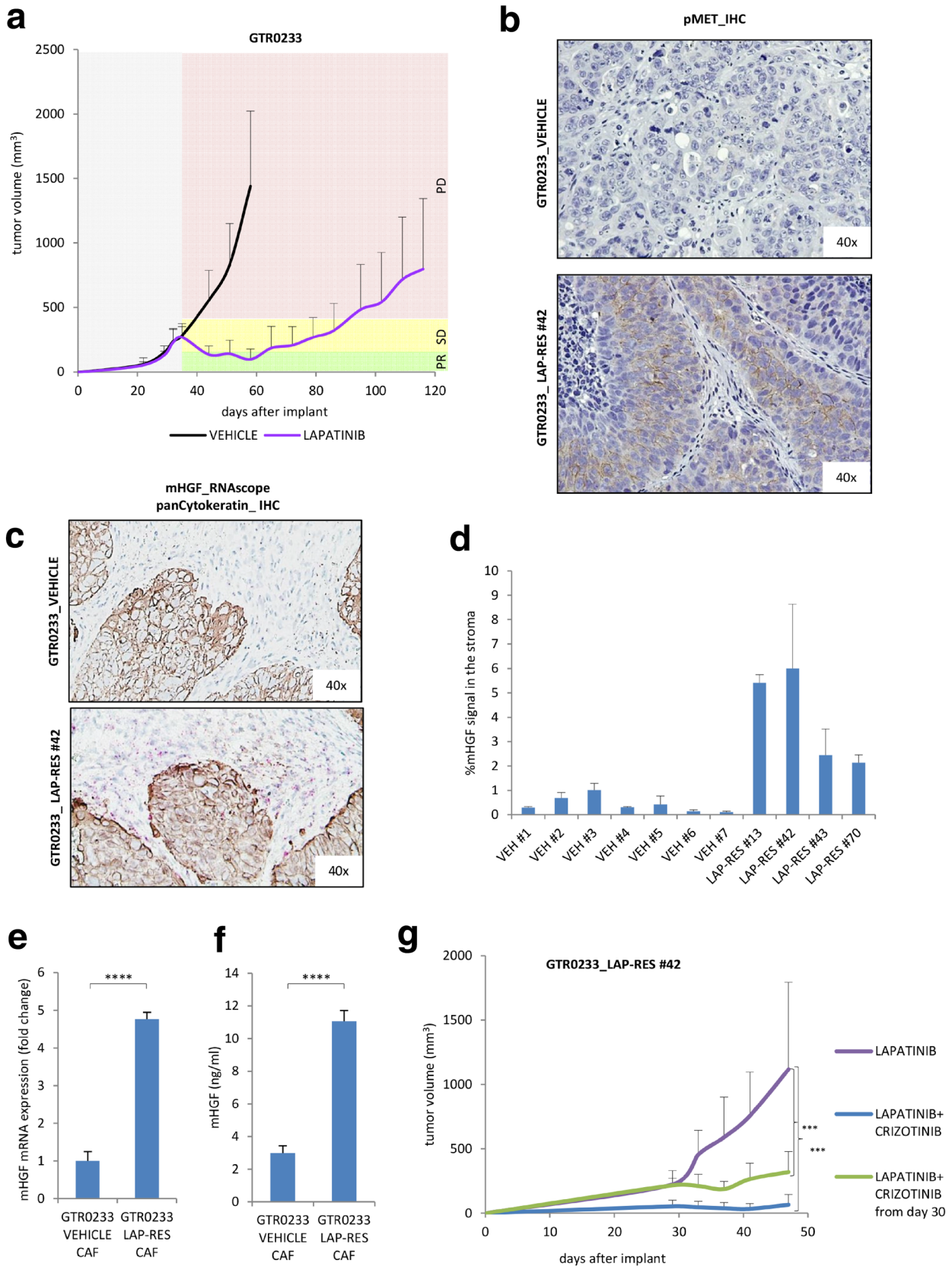
Gomez-Martin and colleagues showed that higher level of *HER2* amplification significantly predicts increased benefit from trastuzumab-based therapy in patients with advanced GC [16]. A mean *HER2*/*CEP17* ratio of 4.7 was found as the optimal cutoff value identifying tumors where *HER2* acts as a driver 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).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GTR0233
tumor volume (mm³)
2500
2000
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500
0
0
20
40
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80
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days after implant
PD
SD
PR
VEHICLE
LAPATINIB
b
pMET_IHC
GTR0233_VEHICLE
40x
GTR0233_ LAP-RES #42
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c
mHGF_RNAscope
panCytokeratin_ IHC
GTR0233_VEHICLE
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d
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10
9
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VEH #1
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VEH #6
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LAP-RES #13
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LAP-RES #43
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e
mHGF mRNA expression (fold change)
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GTR0233 VEHICLE CAF
GTR0233 LAP-RES CAF
f
mHGF (ng/ml)
14
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10
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2
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GTR0233 VEHICLE CAF
GTR0233 LAP-RES CAF
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tumor volume (mm³)
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days after implant
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LAPATINIB
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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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