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## BRCA2 Germline Mutations Identify Gastric Cancers Responsive to PARP Inhibitors

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(Article begins on next page)

**BRCA2 Germline Mutations Identify Gastric Cancers Responsive to PARP Inhibitors**



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**ABSTRACT**

Despite negative results of clinical trials conducted on the overall population of patients with gastric cancer, PARP inhibitor (PARPi) therapeutic strategy still might represent a window of opportunity for a subpopulation of patients with gastric cancer. An estimated 7% to 12% of gastric cancers exhibit a mutational signature associated with homologous recombination (HR) failure, suggesting that these patients could potentially benefit from PARPi. To analyze responsiveness of gastric cancer to PARPi, we exploited a gastroesophageal adenocarcinoma (GEA) platform of patient-derived xenografts (PDX) and PDX-derived primary cells and selected 10 PDXs with loss-of-function mutations in HR pathway genes. Cell viability assays and preclinical trials showed that olaparib treatment was effective in PDXs harboring *BRCA2* germline mutations and somatic inactivation of the second allele. Olaparib responsive tumors were sensitive to oxaliplatin as well. Evaluation of HR deficiency (HRD) and mutational signatures efficiently stratified responder

and nonresponder PDXs. A retrospective analysis on 57 patients with GEA showed that *BRCA2* inactivating variants were associated with longer progression-free survival upon platinum-based regimens. Five of 7 patients with *BRCA2* germline mutations carried the p.K3326\* variant, classified as “benign”. However, familial history of cancer, the absence of RAD51 foci in tumor cells and a high HRD score suggest a deleterious effect of this mutation in gastric cancer. In conclusion, PARPi could represent an effective therapeutic option for *BRCA2*-mutated and/or high HRD score patients with GEA, including patients with familial intestinal gastric cancer.

**Significance:** PARP inhibition is a potential strategy for treating patients with gastric cancer with mutated *BRCA2* or homologous repair deficiency, including patients with familial intestinal gastric cancer, for whom *BRCA2* germline testing should be recommended.

**Introduction**

Despite the efforts spent in translational and clinical research to identify novel molecular targets and develop new therapeutic strategies, gastroesophageal adenocarcinoma (GEA) has a major impact on global health (1). Due to the absence of early symptoms, most cases are diagnosed at late stages and patients’ outcome is still unsatisfactory, with 70% of patients dying of the disease within 5 years.

From a histologic point of view, GEAs are classified based on the Lauren criteria into diffuse and intestinal adenocarcinomas. In 2014, The Cancer Genome Atlas (TCGA) released a comprehensive genomic characterization of these tumors (2) that allowed a molecular classification of GEAs into four major subtypes: (i) CIN: tumors presenting Chromosomal Instability, that account for >50% of all GEAs and are characterized by gross genomic alterations; (ii) MSI: tumors with Microsatellite Instability, endowed with a high mutation rate due to defects in the Mismatch Repair machinery; (iii) GS: Genomically

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69 Stable tumors without CIN and MSI traits; and (iv) EBV-positive:  
70 associated to Epstein-Barr Virus infection.

71 The approved therapeutic options for GEAs are limited, with  
72 surgery and systemic chemotherapy based on the combination or  
73 sequence of various chemotherapy agents (platinum agents, fluoro-  
74 pyrimidines, taxanes, irinotecan, and trifluridine/tipiracil) as mainstay  
75 of care. Regarding targeted therapies, trastuzumab is approved in the  
76 first-line in association to chemotherapy for unresectable or metastatic  
77 HER2-positive gastric cancer. Ramucirumab (targeting VEGFR2) can  
78 be used alone or in combination with paclitaxel in the second-line  
79 setting. However, the addition of trastuzumab increases objective  
80 response rate of only 12% compared with chemotherapy alone  
81 (47% vs. 35% with chemo alone; ref. 3); a similar clinical improvement  
82 is observed when considering ramucirumab (28% vs. 16%; ref. 4).  
83 Regarding immune checkpoint inhibitors, pembrolizumab in combina-  
84 tion with trastuzumab and chemotherapy achieved promising  
85 preliminary activity (5), and has been granted accelerated approval  
86 by FDA. FDA has also approved nivolumab plus chemotherapy as  
87 first-line treatment for advanced metastatic gastric cancer, with a  
88 subset of patients who achieved long-term benefit (6). Despite such  
89 advances and the current development of promising therapeutic  
90 strategies, there are a number of molecular subgroups with low  
91 prevalence, but potential actionability that are at risk of being  
92 neglected.

93 A still open and debated question is whether PARP inhibitors  
94 (PARPi), which are approved for other tumor types such as breast,  
95 ovarian, prostate, and pancreatic cancer (7), may still represent a  
96 potentially valuable option for patients with GEA (8). Indeed, the  
97 percentage of patients showing alterations in DNA double-strand  
98 break (DSB) repair and homologous recombination deficiency (HRD),  
99 who might potentially benefit from PARP inhibition, is relevant:  
100 around 18% in esophageal adenocarcinoma (9) and 7% to 12% in  
101 gastric cancer (10). Unfortunately, clinical trials conducted in patients  
102 with molecularly unselected gastric cancer to address this hypothesis  
103 provided negative results (11), further suggesting that patients' selection  
104 based on HRD is mandatory to potentially achieve treatment  
105 benefit.

106 On these premises, taking advantage of a proprietary annotated  
107 platform of GEA patient-derived xenografts (PDX), we studied the  
108 response to PARPis by performing preclinical trials on gastric cancer

110 PDXs, with the aim of identifying sensitive tumors and discovering  
111 genetic alterations useful for their selection. We identified patients  
112 characterized by germline mutations in the *BRCA2* gene and loss of the  
113 wild-type (WT) allele as optimal candidates for a therapeutic strategy  
114 with PARPi in gastric cancer.

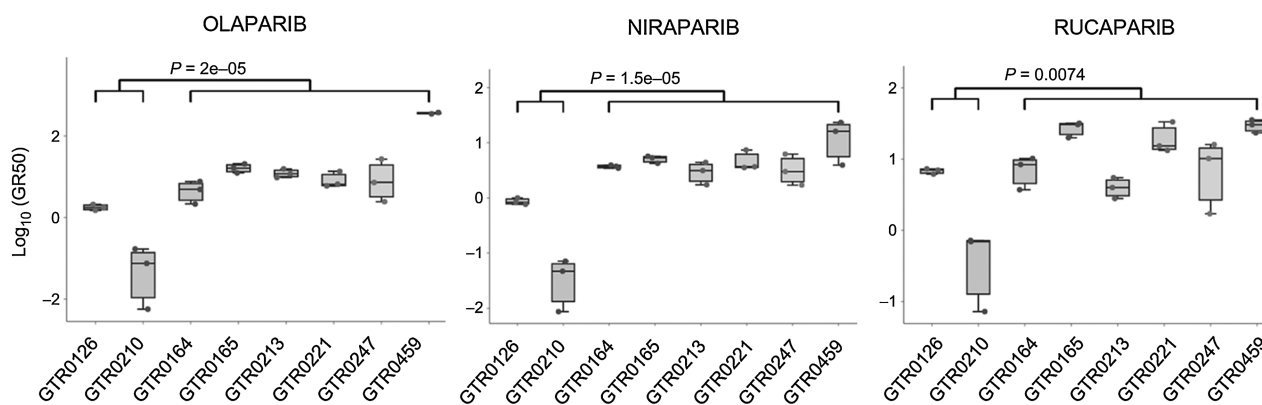
## 115 Materials and Methods

### 116 Primary cell preparation, dose-response cell viability assays 117 and GR50 evaluation

118 Primary cells used in *in vitro* experiments were obtained from GEA  
119 PDX specimens following the procedure described in (12) and main-  
120 tained in culture in Iscove's medium supplemented with 10% FBS and  
121 antibiotics. Genetic identity between primary cells and the original  
122 tumor was verified by short tandem repeat profiling (Cell ID, Pro-  
123 mega); the presence of the indicated gene mutations was confirmed by  
124 Sanger sequencing. *Mycoplasma* testing was routinely performed using  
125 the PCR Mycoplasma Detection Kit (Applied Biological Materials Inc.,  
126 Richmond, BC, Canada). Verified cells are generally thawed few weeks  
127 before the experiments and kept in culture for 3 to 6 months. In all the  
128 experiments, cell viability was assessed by using the CellTiter-Glo  
129 Luminescent Cell Viability Assay (Promega) which measures the ATP  
130 content of the cells. Primary cells were seeded in 96-well plates (3,000-  
131 5,000 cells/well) and cultured in the presence of increasing concentra-  
132 tions of PARPis (1.25-40  $\mu\text{mol/L}$  concentration range for olaparib and  
133 rucaparib; 0.312-10  $\mu\text{mol/L}$  concentration range for niraparib) for  
134 6 days. In Fig. 1, we compared PARPi response in the different models  
135 using the Growth Rate Inhibition 50 (GR50) method that, besides  
136 normalizing to the plating efficiency, also considers the doubling time  
137 of the cells. This computation is recommended when comparing cells  
138 endowed with very different proliferation rates that could confound  
139 the pharmacologic effect (13). The GR50 was calculated starting from  
140 dose-response data using the GRcalculator tool (<http://www.grcalculator.org>) as described in (14). Olaparib, rucaparib, and niraparib were  
141 purchased from Selleckchem. 142

### 143 Preclinical trials in PDXs

144 Experiments were performed on 8 weeks old female immunocom-  
145 promised NOD/SCID mice (Charles River). GTR0210, GTR0126,  
146 GTR0222, GTR0264, GTR0324, GTR0459, GTR0503, and GTR0213



147 **Figure 1.**

148 GEA primary cells bearing *BRCA2* germline mutations and loss of the WT allele are sensitive to PARPis. Boxplots showing the GR50 of primary cells derived from  
149 gastric cancer PDXs exposed to 3 different PARPis: olaparib, niraparib, and rucaparib. Boxes indicate the median  $\pm$  standard deviation of GR50 values of 3  
150 independent dose-response experiments (dots). GR50 and statistical significance (Wilcoxon rank-sum test) were calculated using the GRcalculator tool [(14); see  
151 Methods for details].

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|     |   |     |
|-----|---|-----|
| 149 | PDXs were expanded for 2 to 3 generations to obtain 5 to 7 mice per   | 209 |
| 150 | treatment arm. The presence of the indicated gene mutations was   | 210 |
| 151 | verified by gDNA sequencing for each model before PDX expansion.  | 211 |
| 152 | When tumors reached an average volume of 220 to 250 mm <sup>3</sup> mice were                                     | 212 |
| 153 | randomized and treated for the indicated days with either vehicle   | 213 |
| 154 | (saline) or 100 mg/kg olaparib [5 days/ week, per overall survival (OS)]  |     |
| 155 | or 5 mg/kg oxaliplatin (once/week for 3 weeks, IP). Tumor size was  |     |
| 156 | evaluated once weekly by caliper measurements and approximate   |     |
| 157 | volume of the mass was calculated using the formula $4/3\pi(D/2)(d/2)^2$ ,  |     |
| 158 | where <i>D</i> and <i>d</i> are the major and minor tumor axes, respectively.                                     |     |
| 159 | As often done in PDX models, the response in mice has been evaluated  |     |
| 160 | using RECIST 1.1-like criteria, i.e., progressive disease (PD): $\geq 35\%$                                       |     |
| 161 | increase from baseline; partial response (PR): $\geq 50\%$ reduction from   |     |
| 162 | baseline; stable disease (SD): intermediate variations from base-   |     |
| 163 | line (15). Statistical analysis was performed with GraphPad PRISM   |     |
| 164 | 8.0, using the two-way ANOVA Bonferroni corrected method.   |     |
| 165 | Statistical significance: ns, not significant; *, $P < 0.05$ ; **, $P < 0.01$ ;                                   |     |
| 166 | ***, $P < 0.001$ ; ****, $P < 0.0001$ . Olaparib for <i>in vivo</i> experiments was                               |     |
| 167 | purchased from Biosynth Carbosynth Ltd. Oxaliplatin was kindly  |     |
| 168 | provided by the Hospital Pharmacy. No a priori criteria were used   |     |
| 169 | for including and excluding animals, experimental units or data points;   |     |
| 170 | no confounders were controlled.   |     |
| 171 | <b>Evaluation of HRD score and mutational signatures in PDXs</b>  |     |
| 172 | Genomic DNA extracted from PDX models was captured with   |     |
| 173 | Agilent SureSelect XT Human All Exon V6 (Agilent Technologies,  |     |
| 174 | Santa Clara, CA) and Illumina Exome Panel – enrichment oligos   |     |
| 175 | (Illumina Inc., San Diego, CA) covering 45 Mb of exonic content;  |     |
| 176 | libraries were subjected to paired-end sequencing on Illumina   |     |
| 177 | NextSeq500 and NovaSeq (Illumina, San Diego, CA) producing  |     |
| 178 | 150-bp reads. Raw data were deposited in the EGA Archive  |     |
| 179 | (EGAS00001006790). Reads were aligned to a concatenated   |     |
| 180 | human-mouse genome reference (hg38-mm10) with BWA (16)  |     |
| 181 | and subsequently processed with GATK (17) public best practice  |     |
| 182 | workflows for duplicate removal and base quality recalibration.   |     |
| 183 | After the removal of reads mapping to murine chromosomes,   |     |
| 184 | somatic single-nucleotide variants and Insertion/Deletions were   |     |
| 185 | identified using Mutect2 and annotated with Annovar (18).   |     |
| 186 | Sequenza (19) was used to detect somatic copy-number alterations  |     |
| 187 | (SCNA). Genomic HRD signatures were estimated using   |     |
| 188 | scarHRD (20) from SCNAs and sigLASSO (21) to assign COSMIC  |     |
| 189 | mutational signatures version 2 (22) and Somatic Signatures from  |     |
| 190 | Secrier and colleagues (9) using passing filter mutations from  |     |
| 191 | Mutect2 as input.   |     |
| 192 | <b>RAD51 foci assay</b>   |     |
| 193 | Immunofluorescence stainings were performed as described  |     |
| 194 | in (23) at Vall d'Hebron Institute of Oncology with the antibodies  |     |
| 195 | described in the Supplementary Methods. Biomarkers were quan-   |     |
| 196 | tified on formalin-fixed, paraffin-embedded (FFPE) patient tumor  |     |
| 197 | samples by scoring the percentage of geminin-positive cells with 5  |     |
| 198 | or more nuclear foci. Geminin is a master regulator of cell-cycle   |     |
| 199 | progression that enables to mark for S–G <sub>2</sub> -cell cycle phase (24).                                     |     |
| 200 | Scoring was performed onto live images using a 60x-immersion oil  |     |
| 201 | lens. One hundred geminin-positive cells from at least three rep-   |     |
| 202 | resentative areas of each sample were analyzed. Samples with low  |     |
| 203 | $\gamma$ H2AX (<25% of geminin-positive cells with $\gamma$ H2AX foci) or with                                    |     |
| 204 | <40 geminin-positive cells were not included in the analyses, due to  |     |
| 205 | insufficient endogenous DNA damage or tumor cells in the S–G <sub>2</sub> -                                       |     |
| 206 | phase of the cell cycle, respectively. Scoring was performed twice  |     |
| 207 | using the microscope Nikon TiE at the University of Parma. RAD51  |     |
|     | score was defined as the number of geminin-positive cells that  | 209 |
|     | express more than 5 RAD51 nuclear foci. The predefined cutoff of  | 210 |
|     | 10% for the RAD51 score was used to qualify tumors as HRD   | 211 |
|     | ( $\leq 10\%$ ) or homologous recombination proficient (HRP, $>10\%$ ;  | 212 |
|     | ref. 25).   | 213 |
|     | <b>Patients</b>   | 214 |
|     | Patients included in the clinical dataset had metastatic gastric or   | 215 |
|     | gastroesophageal junction cancers and were treated with platinum-   | 216 |
|     | and fluoropyrimidine-based chemotherapy at Fondazione IRCCS   | 217 |
|     | Istituto Nazionale dei Tumori of Milano. In patients with HER2-   | 218 |
|     | positive disease, trastuzumab was added to doublet chemotherapy as  | 219 |
|     | per standard practice. Pretreatment FFPE tumor samples obtained for   | 220 |
|     | diagnostic purpose were molecularly profiled by means of Foundation   | 221 |
|     | One CDx test. All patients provided written informed consent.   | 222 |
|     | <b>MLH1 gene editing</b>  | 223 |
|     | Cells ( $2 \times 10^5$ to $3 \times 10^5$ ) were transduced overnight with a Cas9                                | 224 |
|     | encoding lentiviral vector (pKLV2-EF1a-Cas9Bsd-W; Addgene,  | 225 |
|     | 68343), in the presence of 8 $\mu$ g/mL polybrene (Millipore). Lentivi-   | 226 |
|     | rus-containing medium was refreshed with complete medium the  | 227 |
|     | following day. Positively infected cells were selected with 20 $\mu$ g/mL   | 228 |
|     | blasticidin (Thermo Fisher Scientific, A1113903), starting 48 hours   | 229 |
|     | after cell transduction. A subsequent infection with the lentiviral   | 230 |
|     | vector pKLV.hygro.cddb_3173 (Kindly provided by Drs. G. Picco and   | 231 |
|     | M. Garnett) containing a single-guide RNA (sgRNA) targeting <i>MLH1</i>   | 232 |
|     | exonic region (GCTACCCAATGCCTCAACCG) was done. Hygro-   | 233 |
|     | micin (500 $\mu$ g/mL; Invitrogen, 10687010) was used to select infected  | 234 |
|     | cells. To identify <i>MLH1</i> -knockout (KO) clones, infected populations  | 235 |
|     | were single-cell cloned in 96-well plates; at least 30 clones were  | 236 |
|     | expanded and analyzed. Gene inactivation was ascertained by Western   | 237 |
|     | blot analysis.  | 238 |
|     | <b>Sanger sequencing of homologous recombination genes</b>  | 239 |
|     | Genomic DNA was extracted from PDXs or primary gastric car-   | 240 |
|     | cinoma cells with Reliaprep gDNA Miniprep system (Promega) or   | 241 |
|     | QIAamp DNA Mini kit (Qiagen) respectively, according to the   | 242 |
|     | manufacturer's instructions. RNA was extracted with RSC miRNA   | 243 |
|     | tissue kit (Promega) and retrotranscribed to cDNA with the High   | 244 |
|     | capacity cDNA retrotranscription kit (Applied Biosystems). The  | 245 |
|     | region of interest was amplified by PCR with the primers reported   | 246 |
|     | in the Supplementary Methods. p.R2336C and ATM were analyzed on   | 247 |
|     | cDNA; all the other mutations on gDNA. The DNA region of interest   | 248 |
|     | was sequenced with Sanger standard method.  | 249 |
|     | <b>IHC</b>  | 250 |
|     | MMR proteins were probed by IHC with antibodies raised against  | 251 |
|     | MLH1 (G168–15, BD Biosciences), MSH2 (FE11, Calbiochem,   | 252 |
|     | Merck), MSH6 (44, BD Biosciences), and PMS2 (A16–4, BD Bios-  | 253 |
|     | ciences). Pathologist reviewed the IHC slides, providing the presence   | 254 |
|     | or not of positive tumor cells showing MMR expression.  | 255 |
|     | <b>Statistics</b>   | 256 |
|     | GR50 was calculated starting from dose–response data using the  | 257 |
|     | GRcalculator tool ( <a href="http://www.grcalculator.org">http://www.grcalculator.org</a> ) as described in (14). | 258 |
|     | For PDX trials, statistical significance was calculated using the two-way   | 259 |
|     | ANOVA with Bonferroni correction.   | 260 |
|     | <b>Study approval</b>   | 261 |
|     | The generation of the GEA PDX platform used in this study and the   | 262 |
|     | molecular and genomic characterization thereof have been extensively  | 263 |

266 described in (26). All animal procedures adhered to the “Animal  
 267 Research: Reporting of In Vivo Experiments” (ARRIVE) standards  
 268 and were approved by the Ethical Commission of the Candiolo Cancer  
 269 Institute (Candiolo, Torino, Italy), and by the Italian Ministry of  
 270 Health (authorization n. 58/2021PR). All patients provided written  
 271 informed consent; samples were collected, and the study was con-  
 272 ducted under the approval of the review boards of all the institutions.  
 273 The study was done in accordance with the principles of the Decla-  
 274 ration of Helsinki, the International Conference on Harmonization,  
 275 and Good Clinical Practice guidelines and GDPR (General Data  
 276 Protection Regulation).  
 277 PDX models data and metadata will be openly available in PDX  
 278 Finder (<https://doi.org/10.1093/nar/gky984>; [pdxfinder.org](https://pdxfinder.org)) and in the  
 279 EurOPDX data portal (<http://dataportal.europdx.eu>) that will be  
 280 updated with the newly generated models.

281 **Data availability statement**

282 The exome sequencing data generated in this study are publicly  
 283 available in EGA Archive (EGAS00001006790). Other raw data gen-  
 284 erated in this study are available upon request from the corresponding  
 285 author.

286 **Results**

287 **GEA primary cells carrying BRCA2 germline mutations and loss**  
 288 **of the WT allele are sensitive to PARP inhibition in vitro**

289 We exploited a proprietary annotated platform of human GEA  
 290 PDXs (26), to analyze their responsiveness to PARPis and unveil  
 291 molecular predictors of treatment benefit. We started with a candidate  
 292 gene approach and searched for GEA models carrying genetic altera-  
 293 tions in genes of the homologous recombination (HR) pathway, some  
 294 of which had been previously correlated with pharmacologic response  
 295 in patients with ovarian cancer (*BRCA1*, *BRCA2*, *ATM*, *ATR*, *BRIP1*,  
 296 *CDK12*, and *PALB2*; refs. 13, 27). We focused our attention only on  
 297 loss-of-function (LOF) mutations, such as nonsense and frameshift  
 298 variants that introduced a premature STOP codon in the protein. Of  
 299 165 genomically annotated PDXs, we selected 6 mutated models  
 300 (GTR0126, GTR0164, GTR0210, GTR0213, GTR0247, GTR0459) for  
 301 which PDX-derived primary cells were available for *in vitro* experi-  
 302 ments (see **Table 1**). The most frequently mutated HR genes were  
 303 *BRCA2* and *ATM* with 4 and 2 LOF models, respectively. One *BRCA2*-  
 304 mutated PDX (GTR0164) also presented LOF mutations in *PALB2* and  
 305 *CDK12*. *In vitro* viability assays were performed on these 6 primary cell  
 306 models and, as negative controls, on primary cells derived from PDXs  
 307 not exhibiting HR gene mutations (GTR0165 and GTR0221). Cells  
 308 were exposed to 3 different clinically approved PARPis—olaparib,  
 309 niraparib and rucaparib—and cell viability was evaluated at increasing  
 310 drug concentrations in dose–response assays. To compare respon-  
 311 siveness, we calculated the GR50 (28), as the proliferation rate and the  
 312 cell doubling time were strikingly different among the different models  
 313 (see Materials and Methods for details). Two models carrying a LOF  
 314 mutation in *BRCA2*, namely GTR0126 and GTR0210, displayed high  
 315 sensitivity to PARPis, especially olaparib and niraparib (**Fig. 1**).  
 316 *BRCA2* LOF variants were also present in GTR0164 and GTR0459  
 317 that showed sensitivity comparable to non-mutated cells (GTR0165  
 318 and GTR0221). When we analyzed more in depth the mutational  
 319 status of *BRCA2*, we found that in GTR0126 and GTR0210 models the  
 320 nonsense mutations were of germline origin, because they were present  
 321 also in the patient’s matched normal gastric mucosa (Supplementary  
 322 Fig. S1). In addition, in both cases the WT allele had undergone loss-of-  
 323 heterozygosity (LOH) in the tumor. On the contrary, the GTR0459

Q7 **Table 1.** HR gene variants and clinical features of GEA models used in the study.

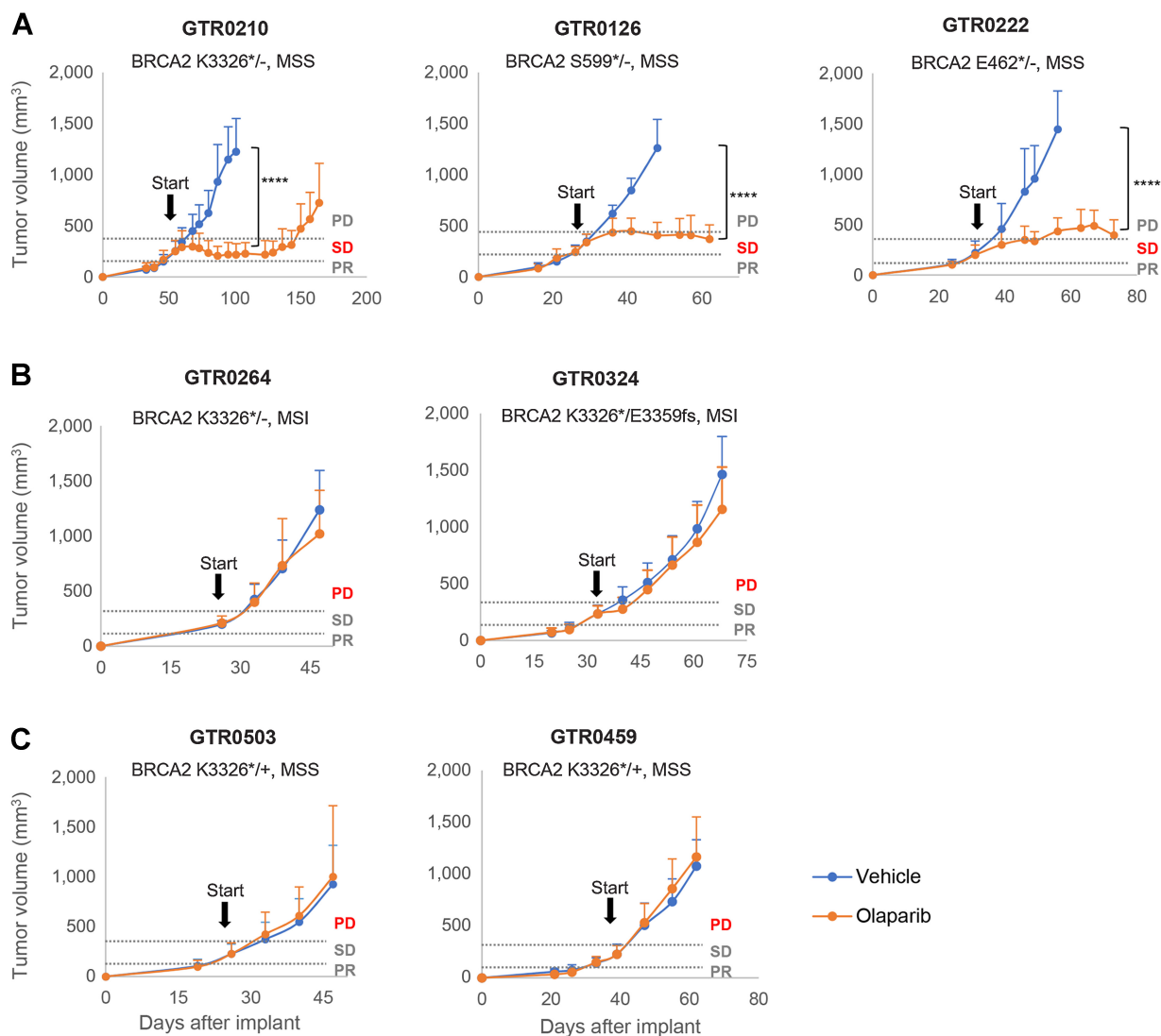
| Tumor ID | HR gene | Variant    | Type of variant | Second hit | Lauren class | MSS/MSI status | Age at diagnosis | Gender | Familial history of cancer   | In vitro models | In vivo models |
|----------|---------|------------|-----------------|------------|--------------|----------------|------------------|--------|--|-----------------|----------------|
| GTR0247  | ATM     | p.S214Pfs  | Frameshift      | NO         | Intestinal   | MSI            | 60               | F      | NA   | ✓               | ✓              |
| GTR0213  | ATM     | p.I1581Nfs | Frameshift      | NO         | Intestinal   | MSI            | 77               | M      | NA   | ✓               | ✓              |
| GTR0164  | BRCA2   | p.K2303Rfs | Frameshift      | NO         | Intestinal   | MSI            | 72               | F      | Gastric k (father, brother)  | ✓               | ✓              |
| GTR0210  | BRCA2   | p.K1691Nfs | Frameshift      | NO         | Intestinal   | MSI            | 43               | M      | Pancreas K (father)  | ✓               | ✓              |
| GTR0264  | BRCA2   | p.K3326*   | Nonsense        | LOH        | Intestinal   | MSI            | 66               | M      | Colon K (proband and father)   | ✓               | ✓              |
| GTR0324  | BRCA2   | p.K3326*   | Nonsense        | p.K3360Nfs | Intestinal   | MSI            | 73               | M      | Gastric K (4 uncles, grandmother), gastric benign disease (brother), breast K (sister) | ✓               | ✓              |
| GTR0459  | BRCA2   | p.K3326*   | Nonsense        | NO         | Intestinal   | MSS            | 74               | F      | Gastric K (father, brother), breast K (sister), lymphoma (daughter)                    | ✓               | ✓              |
| GTR0503  | BRCA2   | p.K3326*   | Nonsense        | NO         | Intestinal   | MSS            | 77               | M      | Larynx K (father), otolaryngo K (brother)  | ✓               | ✓              |
| GTR0126  | BRCA2   | p.S599*    | Nonsense        | LOH        | Intestinal   | MSS            | 77               | M      | Lung K (father), breast K (mother, aunt, daughter, niece)                              | ✓               | ✓              |
| GTR0222  | BRCA2   | p.E462*    | Nonsense        | p.R2336C#  | Intestinal   | MSS            | NA               | M      | NO   | ✓               | ✓              |

In bold: germline variants.  
 #Conflicting interpretation of pathogenicity, according to the ClinVar database  
 NA, not available.

326 PDX, although bearing the same germline *BRCA2* nonsense variant as  
 327 GTR0210, retained the WT allele (Supplementary Fig. S1). In the  
 328 GTR0164 PDX, the identified *BRCA2* mutation was not germline but  
 329 only somatic and the second allele was again WT. Considering these  
 330 results, we hypothesized that GEAs bearing germline inactivating  
 331 mutations in the *BRCA2* gene and loss of the WT allele might be the  
 332 right candidates for PARP inhibition. Interestingly, genetic alterations  
 333 of *ATM* did not seem to confer significant responsiveness to PARPi,  
 334 neither in the presence of a single *ATM* frameshift mutation  
 335 (GTR0247) nor if both alleles were affected (GTR0213; Fig. 1 and  
 336 Supplementary Fig. S1).

### GEA PDXs bearing *BRCA2* germline mutations and loss of the WT allele are responsive to olaparib

To verify responsiveness in patients' tumors we then moved to  
 340 *in vivo* experiments and performed preclinical trials in gastric cancer  
 341 PDXs using olaparib. On the basis of the results obtained in cell  
 342 viability assays, we focused our attention on *BRCA2* germline mutated  
 343 tumors. Besides GTR0126 and GTR0210, already used in *in vitro*  
 344 experiments, in our GEA platform we found 5 additional PDX models  
 345 carrying *BRCA2* deleterious germline mutations, available for xenotrials  
 346 but from which we did not succeed in deriving primary cells for  
 347 *in vitro* assays. In total, 7 *BRCA2* germline mutated PDXs (7 cases of  
 348



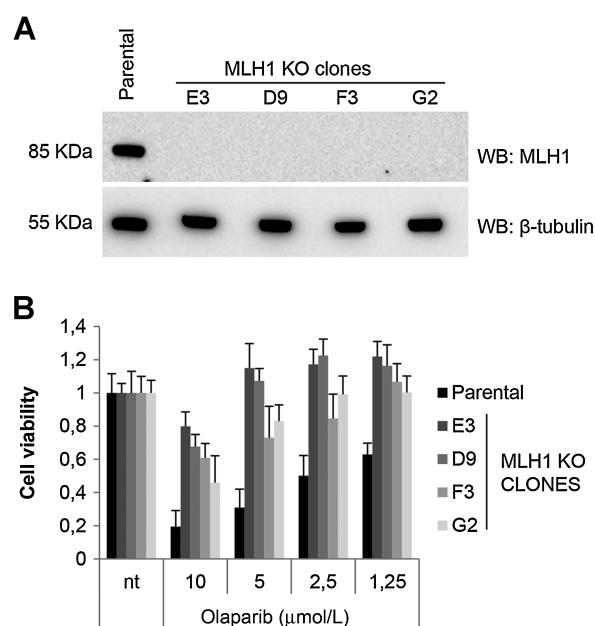
**Figure 2.**

MSS gastric cancers carrying *BRCA2* germline mutations and loss of the WT allele are responsive to olaparib in preclinical trials. Tumor growth curves of the PDX cohorts derived from the *BRCA2* germline mutated human specimens of the indicated models. After reaching an average tumor volume of 220 to 250 mm<sup>3</sup>, PDXs were treated either with placebo (vehicle, blue lines) or olaparib (2 mg/mouse, 5 days/week per OS; orange lines). Lines represent average tumor volume + standard deviation. *N* = 5–7 animals. The response has been evaluated using RECIST 1.1-like criteria: PD: ≥ 35% increase from baseline; PR: ≥ 50% reduction from baseline; SD: intermediate variations from baseline (dashed lines). The clinical response of each PDX is indicated in red. On the top of the graphs the *BRCA2* genotype and the MSS/MSI status of the treated model are indicated; group A comprises *BRCA2* germline mutations and loss of the WT allele in a MSS context; group B shows *BRCA2* germline mutations and loss of the WT allele in a MSI context; group C carries *BRCA2* germline mutations without loss of the WT allele in a MSS context. Arrows = treatment start. Statistical significance was calculated using the Two-way ANOVA with Bonferroni correction. For GTR0126 and GTR0222 the olaparib arm at the end of the trial was compared with the Vehicle arm at the time of mice sacrifice (\*\*\*\*, *P* < 0.0001).

351 165 sequenced PDXs, 4%) were challenged with olaparib. The *BRCA2*  
 352 genotype and clinical characteristics are summarized in **Table 1**. It is  
 353 worth noting that 5 *BRCA2*-mutated patients of 7 carried the same  
 354 germline p.K3326\* truncating variant (GTR0210, GTR0264,  
 355 GTR0324, GTR0459, and GTR0503; Supplementary Figs. S1 and  
 356 S2) and 2 of them (GTR0324 and GTR0459) reported a familial  
 357 history of gastric cancer (**Table 1** and Supplementary Fig. S3). On  
 358 the basis of the Lauren histologic classification, all tumors had  
 359 Intestinal histotype. Most of them were microsatellite stable (MSS),  
 360 whereas GTR0264 and GTR0324 showed microsatellite instability  
 361 (MSI; **Table 1**). All the mutations were confirmed in the PDXs by  
 362 DNA sequencing prior to starting the xenotrials (Supplementary Figs.  
 363 S1 and S2).

364 According to the modified RECIST (26), three models achieved SD  
 365 upon olaparib administration (**Fig. 2**). Among them, the GTR0126  
 366 and GTR0210 models had shown responsiveness also in *in vitro*  
 367 experiments. A third model, GTR0222, for which primary cells for  
 368 *in vitro* assays were not available, achieved SD in the preclinical trial.  
 369 Interestingly this model, besides the germline premature STOP codon  
 370 in *BRCA2* at p.E462\*, as putative second hit bore the somatic missense  
 371 variant p.R2336C (**Table 1** and Supplementary Fig. S2), that has  
 372 conflicting interpretation of pathogenicity in the ClinVar database  
 373 (<https://www.ncbi.nlm.nih.gov/clinvar/variation/96845/>). GTR0264  
 374 and GTR0324 were refractory to PARP inhibition, although in both  
 375 cases the second *BRCA2* allele was inactivated by LOH and a frameshift  
 376 mutation, respectively. As reported for other tumor types, tumor  
 377 growth of GTR0459 and GTR0503 that retained the normal allele  
 378 (Supplementary Figs. S1 and S2) was not affected by olaparib (**Fig. 2**).  
 379 A similar result, confirming the *in vitro* assays, was observed with  
 380 GTR0213 carrying two truncating frameshifts in the *ATM* gene  
 381 (Supplementary Fig. S4), suggesting that *ATM* inactivation might not  
 382 be sufficient to confer responsiveness to PARPi.

383 Trying to explain primary resistance in GTR0264 and GTR0324, we  
 384 hypothesized that it could be associated with the MSI status, which  
 385 could activate molecular mechanisms counteracting PARP inhibition.  
 386 Indeed, GTR0264 and GTR0324 tumors had MSI-high status by  
 387 microsatellite PCR assay (Supplementary Fig. S5A). We also evaluated  
 388 by IHC the expression of different mismatch repair (MMR) genes  
 389 (*MLH1*, *MSH2*, *MSH6*, *PMS2*) and found that the GTR0264 model did  
 390 not express *MSH2*, likely due to a germline truncating mutation (p.  
 391 Q690\*) and a pathogenic splice site variant already reported in Lynch  
 392 Syndrome (c.1511-2A>G, ClinVar VCV000090688.13), while  
 393 GTR0324 lacked *MLH1* and *PMS2* expression (Supplementary  
 394 Fig. S5B). To verify the possible association between a MSI status and  
 395 insensitivity to PARP inhibition, we inactivated in GTR0210 respon-  
 396 sive cells the *MLH1* gene, the MMR gene most frequently lost in MSI  
 397 gastric tumors, by means of genome editing with the CRISPR-Cas9  
 398 system. sgRNAs targeting *MLH1* exonic region were used and multiple  
 399 clones were isolated. Loss of *MLH1* protein expression was confirmed  
 400 by Western blot analysis in 4 clones (**Fig. 3A**). Parental and *MLH1* KO  
 401 cells were grown for 6 days in the presence of increasing doses of  
 402 olaparib. As shown in **Fig. 3B**, *MLH1* inactivation led to loss of drug  
 403 sensitivity. Interestingly, as assessed by PCR assays, at the time of  
 404 experiment execution the *MLH1* KO cells had not yet developed MSI  
 405 (Supplementary Fig. S6). To evaluate whether this was a gastric cancer  
 406 specific effect, we knocked out *MLH1* in CAPAN1, a *BRCA2* mutant  
 407 MMR proficient pancreatic carcinoma cell line sensitive to olaparib  
 408 (Supplementary Fig. S7A), and performed similar assays. Consistently,  
 409 we confirmed the resistance to PARPi upon *MLH1* editing in these  
 410 cells (Supplementary Fig. S7B), suggesting that the interplay between  
 411 HR and MMR is not restricted to gastric cancer cells.



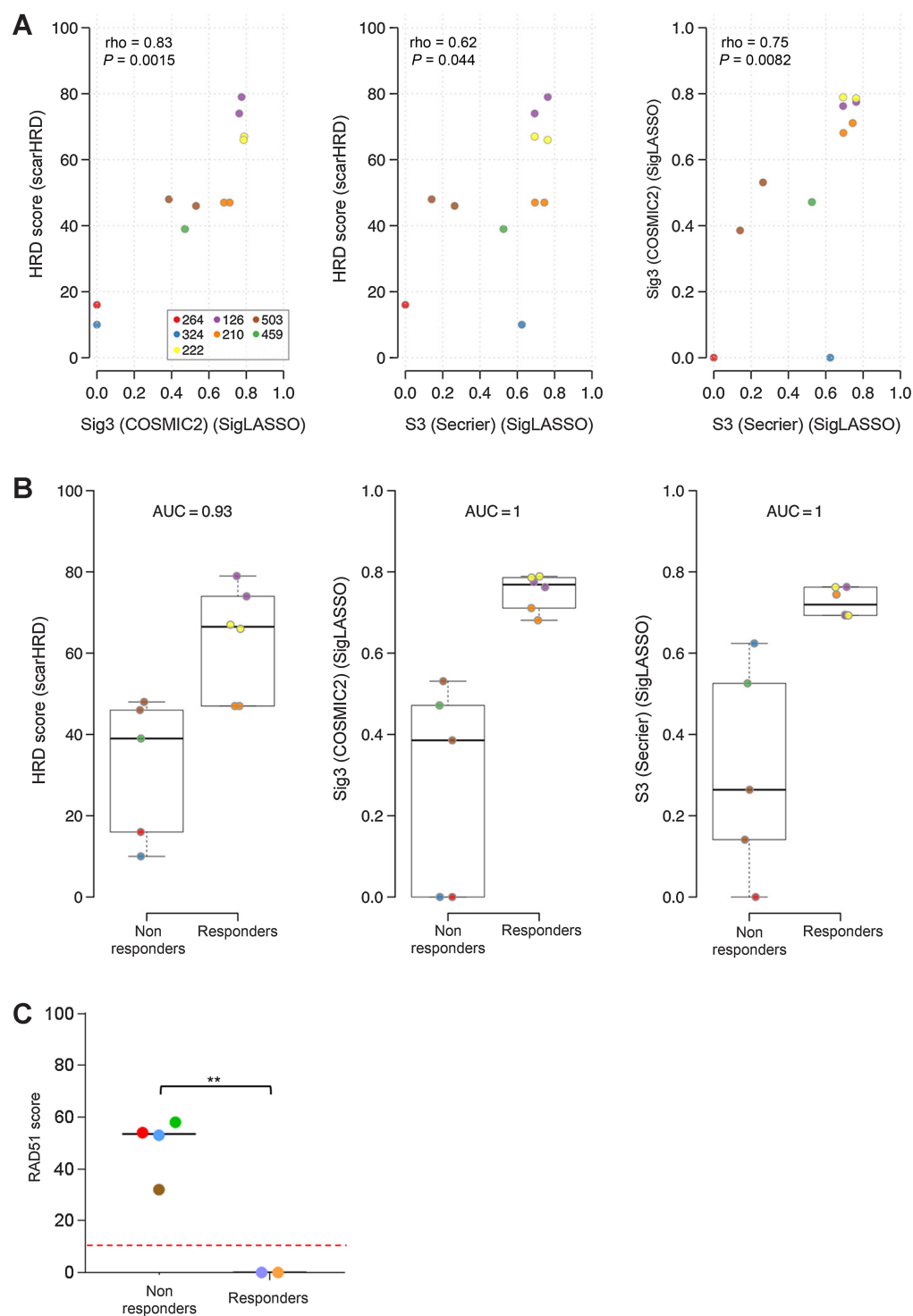
**Figure 3.** MLH1 gene KO abrogates responsiveness to olaparib. **A**, Western blot analysis of 4 different MLH1 KO clones (E3, D9, F3, G2) obtained from GTR0210 primary cells (parental) by CRISPR-Cas9 genome editing. **B**, Cell viability of GTR0210 parental cells and MLH1 KO clones derived thereof, exposed at the indicated increasing concentrations of olaparib for 6 days.

**Genomic HRD signatures predict response to PARPis in GEA**

413 It is generally agreed that HRD could represent a predictor of  
 414 response to PARPis (29) and that the use of HRD testing should enter  
 415 clinical practice for patients' selection (30). Molecular signatures able to  
 416 highlight HRD beyond *BRCA1/2* inactivating mutations have been  
 417 recently described to identify patients with pancreatic and ovarian  
 418 cancer experiencing objective response or longer OS upon platinum  
 419 regimens (31, 32). To evaluate whether a HRD phenotype could be  
 420 informative also for gastric cancer patient selection, we applied different  
 421 tools estimating HRD signatures to the whole-exome sequencing of the  
 422 7 PDXs used in the preclinical trials. We exploited scarHRD (20) and  
 423 SigLASSO (21) based on the COSMIC mutational Signature 3 previ-  
 424 ously found in breast, ovarian, and pancreatic cancers with failure of the  
 425 DNA DSB repair (22) and on the signature S3 reported in HR defective  
 426 esophageal adenocarcinomas (9). Overall, we observed good concor-  
 427 dance across HRD signature levels estimated by the different tools  
 428 (**Fig. 4A**; Supplementary Table S1). Models that showed sensitivity to  
 429 olaparib were associated with higher values of HRD score and HR  
 430 impaired mutational signatures (**Fig. 4B**). Of note, the two signatures  
 431 estimated by SigLASSO [Signature 3 COSMIC and S3 from (22)]  
 432 provided perfect classification of responsiveness to olaparib (AUC = 1).  
 433

**Low RAD51 foci score is associated with responsiveness to PARPis in GEA**

434 Another technique has been recently proposed to envisage tumor  
 435 patients with sensitivity to PARPis, based on the assessment of RAD51  
 436 foci formation in tumor specimens (23). Because RAD51 is recruited to  
 437 DSBs by *BRCA1/2* upon DNA damage, the amount of RAD51 foci in a  
 438 sample can be used as a marker of a proficient or deficient HR  
 439 machinery. We challenged this method on 6 of the 7 GEA PDX  
 440 models used in preclinical trials, as one of them (GTR0222) was not  
 441  
 442

**Figure 4.**

HRD score and mutational signatures predict responsiveness to olaparib. **A**, Scatter plots showing values of HRD and mutational signature score (colored dots) obtained with the indicated tools in the PDXs used in preclinical trials. For the GTR0126, GTR0210, GTR0222, and GTR0503 models the analysis was performed on two different mice. GTR0126, GTR0210, and GTR0222 = responder PDXs; GTR0264, GTR0324, GTR0459, and GTR0503 = nonresponder PDXs. **B**, Boxplot showing distribution of HRD score, COSMIC Signature 3 and S3 signature from (9) in responder and nonresponder PDXs. **C**, Evaluation of the RAD51 score in the PARPi responsive and resistant models used in the preclinical trials shown in **Fig. 2**. GTR0222 tumor tissue was not evaluable due to technical issues. RAD51 score was defined as the number of geminin-positive cells that express at least 5 RAD51 nuclear foci. The predefined cutoff of 10% (red dashed line) for the RAD51 score was used to qualify tumors as HRD ( $\leq 10\%$ ) or HRP ( $>10\%$ ). \*\*,  $P = 0.005$ .



445 suitable for evaluation. The two evaluable olaparib sensitive models  
 446 (GTR0126 and GTR0210) were endowed with a low RAD51 foci score,  
 447 while all the other tumors showed RAD51 foci levels above the 10%  
 448 threshold (Fig. 4C), suggesting that also scoring for RAD51 foci  
 449 formation on routinely prepared tumor specimens could be an infor-  
 450 mative method for GEA patient selection. Importantly, the GTR0210  
 451 tumor sample bearing the p.K3326\* variant and loss of the WT allele,  
 452 showed lack of RAD51 foci (Supplementary Fig. S8A) reminiscent of  
 453 an impaired HR pathway, strongly suggesting a LOF effect of the  
 454 mutation.

455 Interestingly, while the parental GTR0210 primary cells responsive  
 456 to olaparib were HR-deficient with a RAD51 score below 10%, the  
 457 derived *MLH1* KO clones resulted HR-proficient (Supplementary  
 458 Fig. S8B), suggesting that *MLH1* inactivation may have restored HR  
 459 and rendered the cells insensitive to PARP inhibition.

#### 460 **GEAs sensitive to olaparib are cross-sensitive to oxaliplatin**

461 A well consolidated observation in clinical practice is that tumors  
 462 sensitive to PARPis are also responsive to platinum-based chemo-  
 463 therapy (33, 34). To evaluate whether this is the case also in gastric  
 464 cancers, we performed xenotrials with oxaliplatin in the same PDX  
 465 models described above. Basically, tumors that had displayed disease  
 466 stabilization upon olaparib treatment showed a similar response upon  
 467 oxaliplatin administration (Fig. 5). On the basis of RECIST-like  
 468 criteria, nonresponders to olaparib confirmed absence of objective  
 469 response also to oxaliplatin.

#### 470 ***BRCA2*-mutated patients with GEA achieve a prolonged 471 progression-free survival upon platinum-based chemotherapy**

472 Finally, to fuel a potential clinical translation of our preclinical  
 473 observations indicating germline *BRCA2*-mutated tumors lacking the  
 474 WT allele as optimal candidates for PARP inhibition, we assembled a  
 475 cohort of patients with advanced GEA treated at the Fondazione  
 476 IRCCS Istituto Nazionale dei Tumori di Milano with available com-  
 477 prehensive genomic profiling data. Because PARPi are not approved in  
 478 gastric cancer, we considered up-front therapy with platinum agents as  
 479 a surrogate, based on evidence in other tumors (33, 34) and on our  
 480 observation of cross-sensitivity between olaparib and oxaliplatin. We  
 481 included in the analysis 57 patients with advanced GEA treated with  
 482 up-front platinum-fluoropyrimidine regimens (with or without doc-  
 483 etaxel) and with the addition of trastuzumab in HER2-positive disease  
 484 (Supplementary Table S2). The cohort included 4 patients with *BRCA2*  
 485 LOF mutations, 2 of which were germline (see Supplementary  
 486 Table S3). A third patient, with a very early tumor onset, carried the  
 487 p.K3326\* mutation; unfortunately, we did not have suitable material  
 488 available to confirm the germline nature of this variant. It is worth  
 489 noting that 3 of these 4 patients reported a familial history of gastric  
 490 cancer. As shown in the swimmer plot in Fig. 6, patients harboring  
 491 *BRCA2* inactivating variants (red bars) were among the best respon-  
 492 ders, with a progression-free survival (PFS) above the median of  
 493 6.4 months (13.1, 12.5, and 8.0 months). In the same cohort, we also  
 494 evaluated the presence of mutations in genes other than *BRCA1/2*  
 495 involved in the HR machinery including *ATM*, *ATR*, *RAD51*, and  
 496 *FANCA*. Notably, patients bearing deleterious variants in these genes  
 497 (yellow bars) were mostly associated with longer PFS (Fig. 6; Supple-  
 498 mentary Table S3).

## 499 **Discussion**

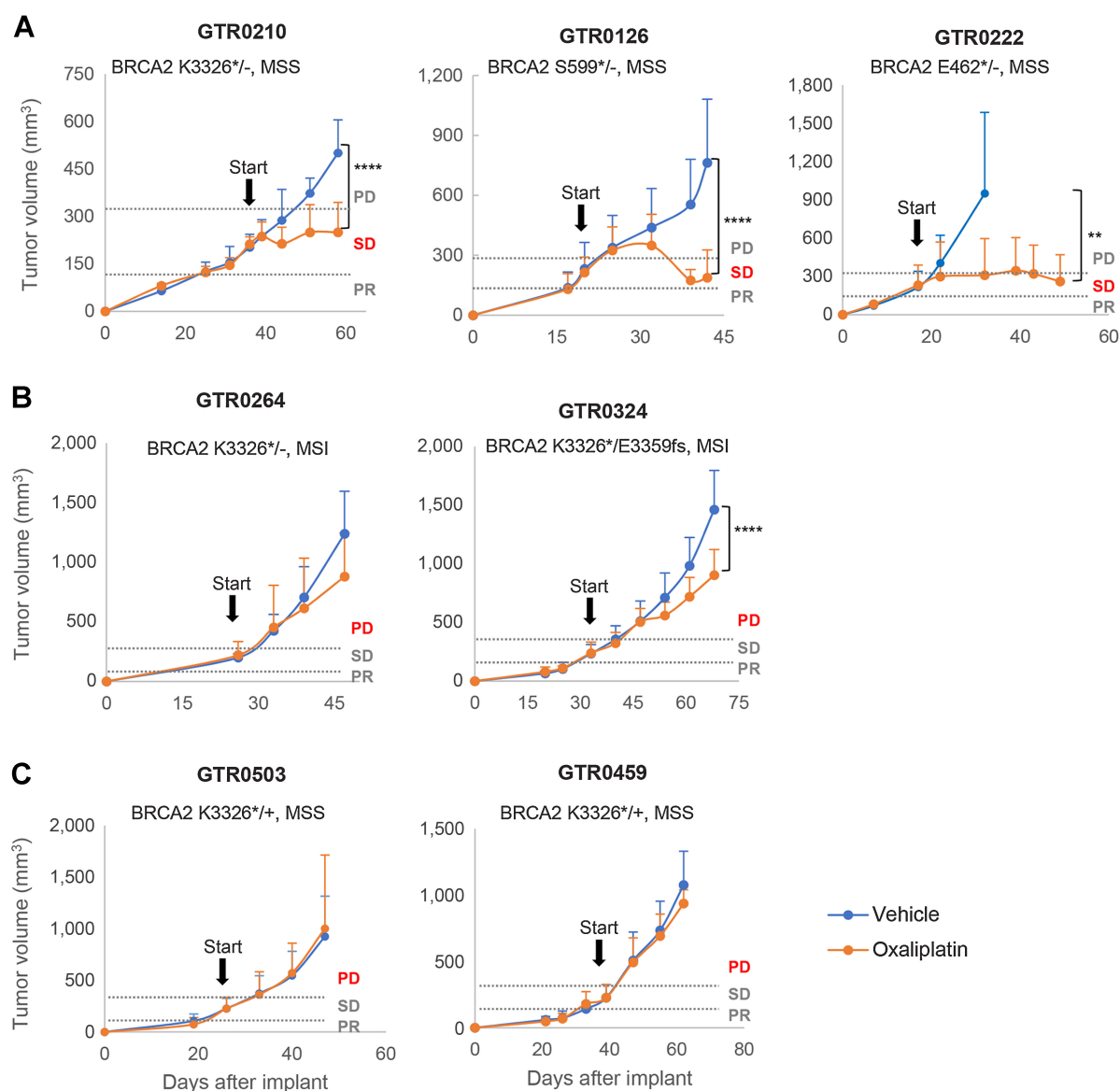
500 GEAs are aggressive and heterogeneous tumors with a 5-year  
 501 survival of less than 20% (1). Because the therapeutic options are

502 limited, in the last years a compelling challenge to find novel phar-  
 503 macologic approaches has guided the efforts of translational and  
 504 clinical gastric cancer research. Great hope was generated by the  
 505 comprehensive genomic characterization in the context of TCGA,  
 506 which allowed the molecular classification of GEA into four molecular  
 507 subtypes (2). However, this knowledge and the multitude of potential  
 508 new targets have been only marginally translated into novel thera-  
 509 peutic opportunities. Currently, the possibility to tailor therapy on  
 510 patients' needs by exploiting specific tumor vulnerabilities, as envis-  
 511 aged by precision medicine, is an unmet medical need for several  
 512 patients' subgroups.

513 A heated debate, instead, accompanies the still open question  
 514 whether an "old" therapeutic strategy, such as inhibition of PARP  
 515 activity in HR-deficient tumors, might be repurposed in GEAs. This  
 516 approach exploits synthetic lethality in tumor cells that have lost the  
 517 mechanisms of HR repair and is already approved for breast, ovarian,  
 518 prostate, and pancreatic cancer. Indeed, 50% of all gastric cancers  
 519 display chromosomal instability (CIN subtype), which is frequently  
 520 related to defects in the HR repair. Around 7% to 18% of GEAs carry  
 521 alterations in the HR pathway (9, 10) and they are catalogued among  
 522 platinum-sensitive ones. Drawing from these assumptions, PARP  
 523 inhibition is potentially a promising therapeutic tool. However, initial  
 524 clinical trials designed to verify this hypothesis were inconclusive: after  
 525 the enthusiasm for the results of a phase II clinical trial comparing  
 526 olaparib+paclitaxel versus paclitaxel alone (35), which highlighted a  
 527 longer OS in patients with ATM-low expressing tumors, the confir-  
 528 matory phase III GOLD trial failed to meet the primary endpoint (11).

529 With the aim to refine the molecular selection of patients potentially  
 530 eligible for PARPi, we exploited our proprietary platform of GEA  
 531 PDXs and started with a candidate gene approach, thus selecting  
 532 models carrying gene alterations in the HR pathway. We limited our  
 533 study to genes whose alterations had already been associated with a  
 534 good response in patients with cancer (8) and found available models  
 535 with inactivating mutations (truncating frameshifts and premature  
 536 STOP codons) in *BRCA2*, *ATM*, *CDK12* and *PALB2*. Detailed analysis  
 537 of genetic alterations unveiled that tumors with sensitivity to PARP  
 538 inhibition, both *in vitro* and in PDX trials, bore germline inactivating  
 539 variants in the *BRCA2* gene and somatic LOH or mutation of the  
 540 second allele. Interestingly, no germline LOF mutation in *BRCA1* was  
 541 found in our GEA PDX platform nor in our patients' cohort, suggest-  
 542 ing that *BRCA1* inactivation is not likely to be significantly involved in  
 543 aetiology of gastric cancer.

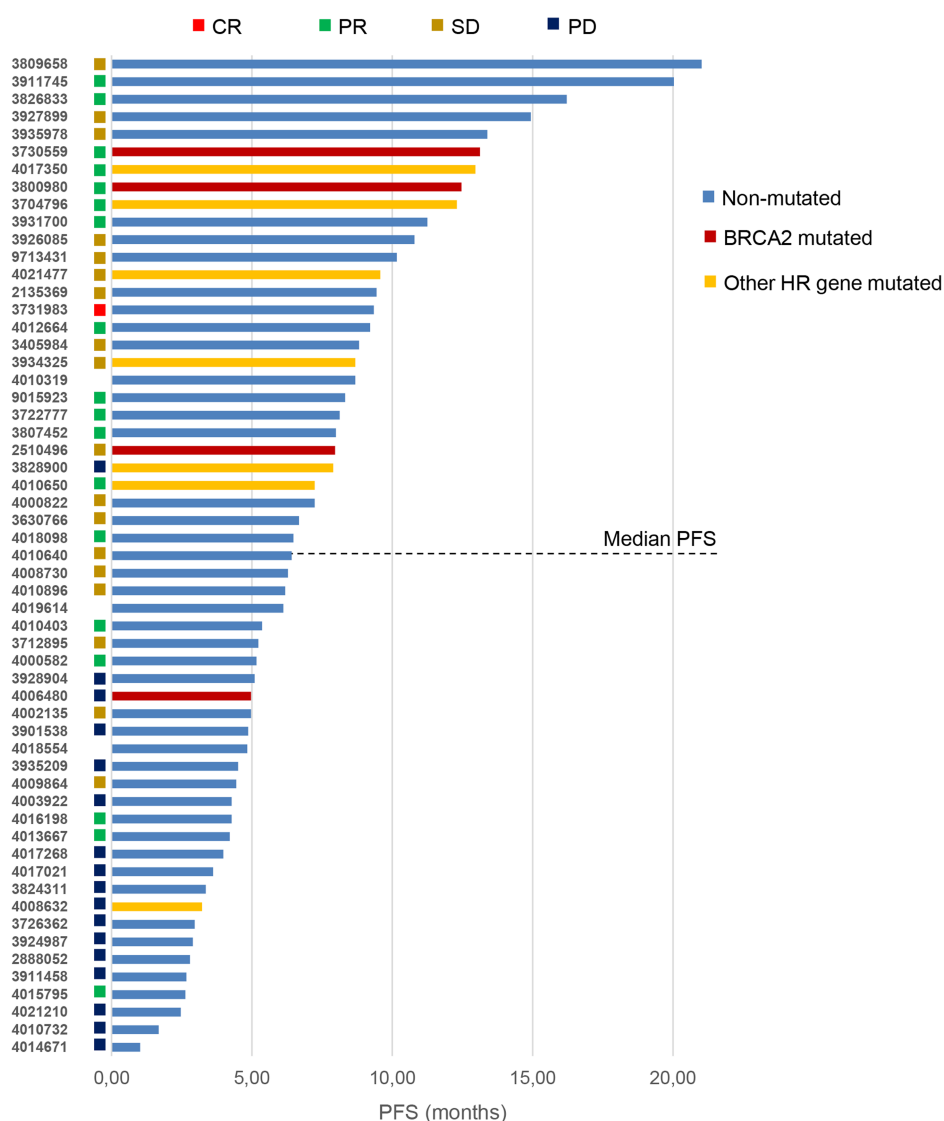
544 From our results, gastric cancer appears to behave as a classic  
 545 BRCA-associated cancer, affected by PARP inhibition when both  
 546 *BRCA2* alleles are functionally inactivated, in accordance with the  
 547 synthetic lethality concept (7, 36). It is worth noting that in our GEA  
 548 PDX platform, 5 of 7 *BRCA2* germline mutated tumors carried the  
 549 same p.K3326\* nonsense mutation. This truncating variant is cur-  
 550 rently classified as "benign" in the ClinVar database (<https://www.ncbi.nlm.nih.gov/clinvar/variation/38266/>). However, the 5 PDX orig-  
 551 inating patients reported a relevant familial history of BRCA-  
 552 associated and nonassociated tumors and two of them (GTR0324 and  
 553 GTR0459) showed a clear familial history of gastric cancer. In addition,  
 554 another patient bearing the p.K3326\* variant, with a familial history of  
 555 gastric cancer and a very early tumor onset (18 years), was indepen-  
 556 dently found in the retrospective patient cohort. Albeit we were not  
 557 able to perform segregation studies to confirm heritability of the p.  
 558 K3326\* in affected relatives due to unavailability of tumor specimens,  
 559 our data suggest that considering this alteration as a neutral poly-  
 560 morphism in the pathophysiology/aetiology of GEAs would be ques-  
 561 tionable. Indeed, the same variant was previously associated to familial  
 562  
 563

**Figure 5.**

Gastric cancer PDXs responsive to olaparib exhibit cross-sensitivity to platinum agents. Tumor growth curves in the same *BRCA2* germline mutated PDX models shown in **Fig. 2**. When reaching an average tumor volume of 220 to 250 mm<sup>3</sup>, mice were treated either with placebo (vehicle, blue lines) or oxaliplatin (0.1 mg/mouse, once a week, IP, for 3 weeks; orange lines). Lines represent average tumor volume + standard deviation. *N* = 4–7 animals. The response has been evaluated using RECIST 1.1-like criteria: PD: ≥ 35% increase from baseline; PR: ≥ 50% reduction from baseline; SD: intermediate variations from baseline (dashed lines). The clinical response of each PDX is indicated in red. On the top of the graphs the *BRCA2* genotype and the MSS/MSI status of the model are indicated (groups A, B, and C as in **Fig. 2**) Arrows = treatment start. Statistical significance was calculated using the two-way ANOVA with Bonferroni correction. For GTR0222 the Oxaliplatin arm at the end of the trial was compared with the Vehicle arm at the time of mice sacrifice (\*\*\*\*, *P* < 0.01).

566 cases of esophageal squamous cell carcinoma (37), pancreatic adeno-  
 567 carcinoma (38), small cell lung cancer, and squamous cell cancer of the  
 568 skin (39). In addition, genome wide association studies demonstrated  
 569 that the p.K3326\* variant is a predisposing factor in lung squamous cell  
 570 carcinoma (40) and upper aerodigestive tract cancer (41). Unlike the  
 571 best known *BRCA2* mutations, p.K3326\* has a mild effect on hormone  
 572 related cancers (39) but is associated with tumors characterized by  
 573 strong environmental genotoxic risk factors, suggesting that affected  
 574 individuals may be more sensitive to genotoxic stress. The p.K3326\* is  
 575 in the last of the 27 exons of the *BRCA2* gene, resulting in the loss of the

93 C-terminal amino acids of the protein (39). Because the RAD51  
 577 interaction domain required for the stabilization of the stalled repli-  
 578 cation forks (42) is very close to this site, it was hypothesized that  
 579 this mutation could interfere with the interaction between *BRCA2* and  
 580 RAD51 (39, 43). Indeed, our observation of the absence of RAD51 foci  
 581 in the GTR0210 PDX tumor specimen confirms this hypothesis,  
 582 suggesting that this variant is less suited to prevent degradation  
 583 of stalled replication forks. This effect could be particularly dan-  
 584 gerous in tissues exposed to genotoxic stress, where replication fork  
 585 progression is continuously challenged. In light of the results  
 586



**Figure 6.** BRCA2-mutated patients with GEA achieve prolonged PFS upon platinum-based chemotherapy. Waterfall plot of PFS in patients with GEA administered platinum agents. Red bars = patients with BRCA2-mutated tumors; yellow bars = patients with LOF mutations in other HR genes (see Supplementary Table S3). The horizontal dashed line indicates the patient with the median PFS (= 6.4 months). CR, complete response.

589 obtained with GTR0222 (*BRCA2* genotype p.E426\*/p.R2336C), it is  
 590 conceivable that also the p.R2336C missense mutation, currently  
 591 annotated with conflicting interpretation of pathogenicity ([https://](https://www.ncbi.nlm.nih.gov/clinvar/variation/96845/)  
 592 [www.ncbi.nlm.nih.gov/clinvar/variation/96845/](https://www.ncbi.nlm.nih.gov/clinvar/variation/96845/)) may be reconsid-  
 593 ered as “likely pathogenic”.

594 Another interesting observation deriving from our studies is that the  
 595 responsiveness to PARPi in GEA seems to be restricted to MSS cancers.  
 596 Indeed, in our preclinical trials, PDXs carrying inactivating alterations  
 597 of both *BRCA2* alleles but characterized by a MSI phenotype  
 598 (GTR0264 and GTR0324) did not respond to treatment. Interestingly,  
 599 KO of the *MLH1* gene through CRISPR/Cas9 genome editing in a  
 600 *BRCA2*-mutated primary cell line responsive to olaparib, was sufficient  
 601 to abrogate the response to treatment. This effect is unlikely due to the  
 602 acquisition of a high mutational burden because the *MLH1* KO cells  
 603 did not exhibit a clear MSI at the time of experiment execution.  
 604 Therefore, olaparib resistance in MSI tumors is not necessarily due to  
 605 the accumulation of mutations typical of the MSI status, but it appears  
 606 causally linked to alterations in the MMR machinery. Indeed we show  
 607 that HR-deficient cells (responsive to PARPi) upon *MLH1* KO not  
 608 only became nonresponsive to PARPi but also reacquired HR profi-

ciency, since they regained the ability to form RAD51 foci. We have  
 also observed that this behaviour is not restricted to gastric cancer cells  
 but can be more general, as we noticed it also in pancreatic cancers  
 cells. Concerning patients, the co-occurrence of inactivation of both  
 HR and MMR is quite rare in most tumors except for prostate  
 cancer (44). Interestingly, Sokol and colleagues found that two patients  
 with prostate cancer with co-occurring *BRCA* and MMR mutations  
 were not responsive to PARPi. Even if it is possible that in MSI tumors  
*BRCA2* mutations are not real drivers (but rather passenger alterations  
 associated with hypermutation status), it is also conceivable that the  
 interplay between the two repair systems might play a role in refrac-  
 toriness to PARPi.

Interestingly, also the PDX carrying 2 inactivating mutations in  
*ATM* (GTR0213) and not responding to olaparib displayed a MSI  
 phenotype. It would be of interest to establish whether gastric cancers  
 bearing inactivating mutations in both *ATM* alleles coupled with MSS  
 status would benefit from PARP inhibition. Unfortunately, our GEA  
 platform did not include suitable models to address this possibility,  
 which remains to be explored in future works. Currently, there is no  
 clear explanation for our experimental observation even though it is

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632 known that HR and MMR are physiologically linked, with MMR  
633 regulating Homeologous Recombination during meiosis (45–47).  
634 Albeit the identification of the mechanism through which MLH1  
635 expression impacts on response to olaparib is out of the aim of this  
636 work, it represents a very interesting field of investigation.

637 In line with the general agreement that HRD might be an agnostic  
638 biomarker of responsiveness to PARPi, genetic analysis of the PDX  
639 models used in preclinical trials revealed that responsive tumors were  
640 associated with high values of HRD score and with *BRCA*-associated  
641 mutational signatures. It is worth noting that the COSMIC Signature  
642 3 (10) and the esophageal adenocarcinoma specific signature S3 (9)  
643 were able to discriminate responder from nonresponder tumors with  
644 surprising accuracy, thus representing a promising tool for GEA  
645 patient selection. Notably, the prevalence of patients with gastric  
646 cancer with HRD identified by Signature 3 was about 7–12% (10).  
647 Thus, the assessment of germline *BRCA* mutations alone may miss a  
648 relevant proportion of patients with platinum-sensitive disease and/or  
649 PARPi sensitivity potentially driven by HRD, but lacking specific  
650 genomic alterations in the HR pathway. In this perspective, perform-  
651 ing a post-hoc analysis of these mutational signatures and/or *BRCA2*  
652 germline mutations in the cohort of patients enrolled in the GOLD trial  
653 would be of outstanding interest to verify their predictive clinical value.  
654 The results obtained with the *in silico* analysis are in line with those  
655 obtained experimentally with the RAD51 foci evaluation. Indeed, high  
656 values of HRD score were associated with low RAD51 scores, suggest-  
657 ing that also the analysis of RAD51 foci formation on routinely  
658 prepared tumor specimens could be an informative method for GEA  
659 patient selection.

660 Our findings support the idea that germline deleterious variants in  
661 the *BRCA2* gene could act as predisposing factors in the development  
662 of GEA, because patients harboring those mutations often reported a  
663 familial history of gastric cancer. GEAs associated to *BRCA2* mutations  
664 are almost invariably classified as intestinal according to the Lauren  
665 classification, indicating that *BRCA2* germline mutations could  
666 account for a percentage of familial intestinal gastric cancer (FIGC)  
667 cases. It is also tempting to speculate that germline LOF mutations in  
668 other classical HR genes such as *ATM* and *PALB2*, albeit rare, might  
669 predispose to the development of FIGC HR-deficient tumors vulner-  
670 able to PARP inhibition. Indeed, a recent study performed on patients  
671 with unselected gastric and esophageal cancer highlighted the presence  
672 of pathogenic germline variants in these genes as well (48). Unfortu-  
673 nately, our GEA PDX platform does not include informative models to  
674 address this issue. In a scenario where the molecular basis of FIGC still  
675 remains unresolved, our results suggest that *BRCA2* germline genetic  
676 testing and PARPi based therapies in positive cases could represent a  
677 new clinical strategy for patient management.

678 In conclusion, we indicate *BRCA2* germline mutated GEAs bearing  
679 loss of the WT allele and MSS traits as optimal candidates for a PARPi  
680 strategy. Clinical trials with PARPi in a proper molecularly selected  
681 GEA patient population would be of outstanding interest to confirm  
682 our preclinical data.

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