

Dual HER2 and TROP2 Antibody–Drug Conjugates Overcome Tumor Heterogeneity in Breast Cancer

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Abstract

Background

Antibody–drug conjugates (ADCs) have transformed the treatment of breast cancer (BC), yet their efficacy remains constrained by heterogeneous target expression and the emergence of resistance. We hypothesized that simultaneous targeting of complementary tumor-associated antigens could broaden tumor-cell coverage and overcome antigen-driven therapeutic escape.

Methods

We investigated combinations of the HER2-directed ADC trastuzumab deruxtecan (T-DXd) with the TROP2-directed ADCs datopotamab deruxtecan (Dato-DXd) or sacituzumab govitecan (S-Gov) in three BC cell lines using matrix-based drug interaction analyses. To define the biological rationale for dual targeting, we characterized HER2 and TROP2 expression and coexpression across 13,664 primary BC tumors using bulk transcriptomic data and at single-cell resolution in 206,347 cancer cells from 112 primary BCs. Protein-level expression and spatial organization were further assessed by multiplex immunofluorescence in eight invasive BC specimens.

Results

T-DXd combined with either Dato-DXd or S-Gov demonstrated synergistic antiproliferative activity across all BC cell lines, including at clinically achievable concentrations. In primary tumors, HER2 and TROP2 expression was complementary, with 47% of tumors expressing at least one target compared with 34% for TROP2 alone. Single-cell analysis revealed marked intratumoral heterogeneity, with HER2-only, TROP2-only, double-positive, and double-negative cancer-cell populations frequently coexisting within individual tumors. Dual targeting increased the proportion of theoretically targetable cancer cells by a median of 13% across individual tumors, with substantial interpatient variability. Multiplex immunofluorescence confirmed these distinct cellular phenotypes at the protein level and revealed heterogeneous spatial organization, including enriched niches and intermixed heterotypic patterns.

Conclusions

HER2 and TROP2 represent complementary therapeutic vulnerabilities within heterogeneous BC ecosystems. Dual HER2/TROP2 targeting may increase both the breadth of tumor-cell coverage and, in double-positive cells, the intensity of ADC-mediated payload delivery. These findings provide a rationale for biomarker-driven ADC combinations based on quantitative single-cell target complementarity and spatial organization rather than conventional binary tumor-level classification. Prospective clinical

studies will be required to determine whether this approach can translate into improved therapeutic efficacy and overcome antigen-driven resistance.

Introduction

Antibody-drug conjugates (ADCs) are a growing class of cancer therapy in which antibodies specific for tumor-associated antigens are used to selectively target the delivery of cytotoxic drugs to cancer cells. They are rapidly changing the therapeutic landscape of oncology [1–5]. To date, four ADCs are marketed in breast cancer (BC): two anti-HER2, ado-trastuzumab emtansine (T-DM1) and trastuzumab deruxtecan (T-DXd), and two anti-TROP2, sacituzumab govitecan (S-Gov) and datopotamab deruxtecan (Dato-DXd). T-DM1, approved for patients with HER2 + mBC in 2013, was then brought in the adjuvant setting. T-DXd, S-Gov, and Dato-DXd, approved for the treatment of mBC, are pushing to the first line of care and are being tested in the (neo)adjuvant setting. Several others (> 140) are under development [6]. However, resistance, either primary or secondary, is inescapable. Different mechanisms are being reported [7–11], including low and/or heterogeneous tumor expression of ADC antigen targets [10, 12–17]. In HER2-low triple negative (TN) advanced BC, S-Gov and T-DXd are used sequentially after progression, but the results are disappointing [18].

Breast cancer is characterized by substantial inter-tumor and intra-tumor heterogeneity at the molecular, cellular, and histological levels [19]. The inter-tumor heterogeneity relies for example on the existence of molecular subtypes defined by expression of hormone receptors (ER and PR) and HER2 and associated with different clinical outcome. Intra-tumor heterogeneity relies on the coexistence within the tumor of diverse cancer cell populations displaying different molecular and phenotypic profiles; it represents a major factor contributing to therapeutic resistance and disease progression [19, 20]. For example, the heterogeneity of ER or HER2 expression has been associated with shorter survival [21, 22]. Antigen target expression is also heterogeneous at the single-cell level within individual tumors, as demonstrated by single-cell RNA-sequencing (scRNAseq) and multiplex immunofluorescence (mIF) approaches [23, 24].

Targeting more than one molecular target (surface antigen, DNA, mitotic spindle, and/or oncogene driver) is one strategy to prevent the tumor resistance and escape [25–27]. Today, multidrug regimens are routinely delivered in clinics in curative situations, and in selected first-line metastatic situations. An increasing number of clinical trials have been testing ADCs in different combinations with other anticancer therapies, such as chemotherapy, endocrine therapy, immune checkpoint inhibitors (ICI), monoclonal antibodies, and small targeted agents, reporting promising results based on possible synergistic effects [28, 29]. For example, combinations with ICIs yielded demonstrable survival advantages in different cancers [6], including BC [30, 31]. Data on dual ADC combinations are very rare. To our knowledge, only one clinical trial has been reported: the DAD (Double Antibody-Drug conjugate) trial in patients with advanced urothelial carcinoma [32]. In this phase I, 24 patients received the combination of Enfortumab-vedotin (EV), anti-nectin-4 ADC, with S-Gov after failure of platinum-based chemotherapy and ICI-based immunotherapy. The authors observed an impressive 70% objective

response rate (ORR), superior to that observed with monotherapy in the pivotal trials (41% for EV and 28% for S-Gov). Using gene expression data, we showed complementary expression and co-expression of nectin-4 and TROP2 in clinical urothelial carcinoma samples and in cancer cells that likely contributed to this high ORR [33]. In BC, one recent pre-clinical study [34] reported a synergic growth inhibitory effect of T-DXd and Dato-DXd combination in three BC cell lines (MCF-7 HER2-OE, 18cS1 HER2-OE, ZR-75-1) and in one 18CS1-derived mouse xenograft. Today, analyses of the bulk and single-cell expression of both HER2 and TROP2 in BC are lacking.

Here, our objective was to reinforce the pre-clinical rationale for anti-HER2 and anti-TROP2 ADCs combinations in BC. We treated three BC cell lines with the combination of T-DXd and Dato-DXd or S-Gov, and then systematically assessed the degree of co-expression of HER2 and TROP2 in clinical samples at the bulk and sc levels.

Materials and Methods

Breast cancer cell lines and ADCs for drug testing

To assess the potential synergy of combining anti-HER2 and anti-TROP2 ADCs, we selected three BC cell lines, SKBR3, SUM190, and HCC1954, that show high gene expression of HER2 and TROP2 (**Figure S1**). The SUM190 cell line was purchased from Biolyt (Germany) and cultured in Ham's F-12 medium supplemented with 2% FBS, non-essential amino acids, 1% ITS-X (Gibco), 6.7 ng/ml triiodo-L-tyrosine, 1% penicillin-streptomycin, and 2 mM glutamine. The SKBR3 and HCC1954 cell lines were cultured in RPMI-1640 supplemented with 10% FBS, 1% non-essential amino acids, 1 mM sodium pyruvate, 0.5% glucose, and 1% penicillin streptomycin. All cell lines are HR-negative. Absence of mycoplasma contamination was regularly verified by PCR (Eurofins Genomics, Germany), and the MycoAlert™ PLUS test (Lonza). T-DXd and S-Gov were obtained from clinics, and Dato-DXd was purchased from MedChemExpress (Sollentuna, Sweden).

Synergy assays between anti-HER2 and anti-TROP2 ADCs

SKBR3 cells were plated at a density of 3,000 cells per well in a 96-well plate (Sarstedt). After 24 hours of incubation, the cells were treated with concentration ranges of T-DXd (0 to 1.5 µg/ml) and Dato-DXd (0 to 15 µg/ml) in the presence of CellTox Green (Promega). Cells were exposed to treatment for 5 days. Fluorescence was acquired every 24 hours using the Cellcyte X live-cell imaging system (Cytena). After 5 days of treatment, growth inhibition was measured using the CellTiter-Glo Cell Viability (Promega) assay on a FluoStar Optima plate reader (BMG Labtech).

SUM190 and HCC1954 cell lines were plated at a density of 3,000 cells per well in a 96-well plate (Sarstedt). After 24 hours of incubation, the cells were exposed for 5 days to cross-ranges of T-DXd (0 to 3 µg/ml) and S-Gov (from 0 to 1.2 µg/ml). Growth inhibition was measured using the Alamar Blue assay.

Consecutive effect analysis of ADC combinations was performed using SynergyFinder software with the Highest Single Agent (HSA) model. The combination effect was compared with the cytotoxicity of each ADC alone. An HSA score superior to + 10 was considered indicative of synergy. Synergy assays were performed in duplicate. For SKBR3 cells, cell mortality curves were generated to compare the time-dependent effects of T-DXd and Dato-DXd as single treatments with their synergistic combination.

Gene expression analysis of HER2 and TROP2

We first assessed the correlation between mRNA and protein expression for these two targets in 363 cancer cell lines, including 30 BC cell lines using the Dependency Map (DepMap) dataset. For both targets, expression levels were positively correlated (**Figure S2**).

Expression analysis was done in untreated clinical BC samples at two resolution levels: bulk and single cell. At the bulk level (“tumor resolution”), we analyzed our BC gene expression database [35] pooled from 44 public data sets (**Table S1**), comprising 13,664 non-redundant non-metastatic, non-inflammatory, primary, and invasive BC samples and 433 normal breast (NB) samples. The gene expression profiles were generated using DNA microarrays or RNA-Seq. These datasets were collected from different sources. Several steps of pre-analytic data processing were applied as previously reported [36]. Before analysis, gene expression levels were standardized within each data set using the PAM50 luminal A population as reference. For tumors without available IHC data, and thanks to the bimodal distribution of respective mRNA expression levels, the ER, progesterone receptor (PR), and HER2 statuses (negative/positive) were defined on transcriptional data of *ESR1*, *PGR*, and *HER2* respectively, as described [37]. The molecular subtypes of samples were defined as HR+/HER2- for ER-positive and/or PR-positive and HER2-negative tumors, HER2+ for HER2-positive tumors, and triple-negative (TN) for ER-negative, PR-negative, and HER2-negative tumors. The co-expression of mRNA expression of ADC targets was assessed on categorical values. The cut-off defining positive *versus* negative expression was defined using ROC curve for each gene as the value maximizing the ROC area in the distinction between the BC and NB samples in the whole population. We then defined the percentages of double-positive *versus* double-negative and simple-positive tumors.

At the single-cell level (“cell resolution”), we analyzed our BC gene expression database pooled from five public data sets (**Table S1**), comprising 112 primary invasive breast cancers [38–42]. For each sample, the gene expression profile was generated using scRNA-Seq. These datasets were obtained from different sources. Epithelial cancer cells were distinguished from epithelial normal cells using the InferCNV algorithm [43]. This latter approach infers the CNV from expression data, allowing computation of a genomic instability score that predicts cancer *versus* normal status. The co-expression of mRNA for ADC targets was assessed on categorical values, using the first quartile as the cut-off for defining positive *versus* negative expression. Each dataset was analyzed separately.

Protein expression analysis of HER2 and TROP2

We analyzed a series of eight invasive ductal BCs collected from non-metastatic (N = 4 breast samples) and metastatic (N = 4: 1 breast, 1 lung, and 2 lymph nodes) patients treated at our institution. FFPE tissue sections (3–4 μm thick) were prepared using a microtome HM340E® and mounted on SuperFrost Plus™ slides (Thermo Fisher Scientific). The slides were air-dried at room temperature for 6 hours and incubated overnight in an oven at 56°C to initiate deparaffinization. The following day, the slides underwent deparaffinization and heat-induced epitope retrieval (Tris-EDTA, pH 9) using the standard PT Link (Dako Agilent) protocol. To prevent tissue degradation, the slides were transferred into 1X PBS before multiplex immunofluorescence (mIF). Background autofluorescence inherent to FFPE samples was quenched by incubating the slides with 0.3% Sudan Black B (SBB; Sigma-Aldrich) in 70% ethanol for 20 minutes. The slides were washed several times with 1X PBS containing 0.02% Tween 20 until all powder deposits were removed. The tissues were permeabilized with 0.5% Triton X-100 in 1X PBS for 15 minutes. Non-specific binding sites were blocked by incubation with a solution containing 5% normal horse serum and 1:1000 azide in 1X PBS for 30 minutes. The primary antibody panel, diluted in the blocking solution, was incubated for 1 hour at room temperature, in a humidified chamber. Nuclei were counterstained with DAPI (1:2000; Thermo Scientific). Secondary antibodies were incubated in a similar manner. Between each labeling step, excess antibody was removed by successive washes with the wash/blocking solution (PNA), followed by 1X PBS and then Milli-Q water, before the samples were dried and mounted using ProLong antifade mounting media (#P36934, ThermoScientific) and a confocal-compatible coverslip (High Precision 18 mm²). The slides were left overnight at room temperature to allow ProLong to polymerize and were then stored at 4°C until image acquisition. The labeled sections were visualized and imaged with a confocal microscope (LSM880®, Zeiss) at 25x magnification with oil immersion, using appropriate exposure times. The characteristics and dilutions of the primary and secondary antibodies used are described in **Table S2**.

The images were subsequently processed using ZEN Blue® software, and examined by two independent cytologists. Tumor cells were identified based on DAPI and Cytokeratin staining. For each marker of interest (TROP2 or HER2), the threshold for positivity was established based on the fluorescence background measured in the corresponding negative/isotype controls. Cells displaying a fluorescence intensity above this threshold were classified as positive for the marker. First, the staining was compared with IHC single staining for the molecule of interest to ensure coherence between the two approaches. Second, the zones with degraded tissue were removed from the area to be analyzed. Third, the individual cells were segmented using the Steinbock toolkit [44] with DAPI as the nuclear channel, and Cytokeratin, HER2 and TROP2 as cytoskeleton/membrane channels. The signal intensities and coordinates for each cell were measured and exported using scikit-image and NumPy in Python (version 3.10.13). The single-cell properties were imported and read as a SpatialExperiment object using the SpatialExperiment package in R (version 4.6.0). Single-cell data and masks were then used for gating based on visualization and distribution using EBImage and Cytomapper. The cells were first classified as Cytokeratin + or Cytokeratin-, then the positivity for TROP2 and HER2 was assessed within Cytokeratin+ cells. The cell neighboring was defined by performing a Delaunay triangulation on cell centroids. The cells that were positive for only one marker of interest (HER2 or TROP2) were considered to belong to a

homogeneous niche when their neighborhood did not contain any cells positive for the other marker of interest, including double-positive cells; otherwise, they were considered to have a heterotypic neighborhood.

Results

Anti-HER2 and anti-TROP2 ADCs combination displays synergic activity in vitro

We assessed the eventual synergistic effect of the T-DXd and Dato-DXd or S-Gov combinations on BC cell lines using SynergyFinder. We first tested SKBR3 (Fig. 1A-C). As shown in Fig. 1A, positive HSA scores were observed after 5 days of treatment across a broad range of concentration pairs. We observed a concentration-dependent synergistic interaction between the two drugs, with the highest synergy scores at intermediate concentrations of both agents, suggesting that the enhanced antiproliferative effect of the combination cannot be explained solely by the activity of either agent individually. The "hot spot" of synergy (maximal HSA score = 18.24) was observed at 1.49 µg/ml of Dato-DXd combined with 0.0153 µg/ml of T-DXd, concentrations that are clinically achievable. The combined treatment reduced cell viability to 19%, *versus* 38% with T-DXd alone and 52% with Dato-DXd alone (data not shown). Such a high HSA score indicated a strong synergistic interaction within this concentration range. Figure 1B illustrates the reproducibility of this synergy score at these drug concentrations across replicates. Figure 1C shows live cell mortality curves measured every 24 hours for five days under different treatments and concentrations. The combination of Dato-DXd (1.49 µg/mL) and T-DXd (0.0153 µg/mL) resulted in a greater increase in cell mortality than either ADC alone given at the same concentration and even at a 10-fold higher concentration. The difference became apparent at 48h and persisted throughout the treatment period. At each time point, the ADC combination generated the highest level of cell mortality among all tested conditions.

We confirmed this synergy in two additional BC cell lines, SUM190 and HCC1954, and by replacing Dato-DXd with another anti-TROP2 ADC, S-Gov (Fig. 1D, 1F). For both cell lines, we observed positive HSA scores across a broad range of concentration pairs and a concentration-dependent synergy between the two drugs, with the highest synergy at intermediate concentrations of both agents. The "hot spot" of synergy was observed at clinically achievable concentrations: approximately 0.048 µg/ml S-Gov combined with 0.0048 µg/ml T-DXd for SUM190 (maximal HSA score = 25.57; Fig. 1D) and 0.08 µg/ml S-Gov combined with 0.096 µg/ml T-DXd for HCC1954 (maximal HSA score = 24.84; Fig. 1F). For SUM190, cell viability was reduced to 28% with the ADC combination, *versus* 69.5% with T-DXd alone, and 54% with S-Gov alone. For HCC1954, the corresponding values were 49%, *versus* 76% and 74%. Here too, reproducibility of synergy was good between replicates (Fig. 1E, 1G).

Altogether, these results indicated a synergistic anti-proliferative effect of anti-HER2 and anti-TROP2 ADC combinations in BC cell lines. Because co-expression of ADC targets may explain this synergy, we then analyzed the targets co-expression.

Gene expression of HER2 and TROP2 in tumors is complementary and ADC combination increases the number of targetable patients.

We analyzed the transcriptomic data from 13,664 BC samples and 433 NB samples. The characteristics of cancer samples are summarized in **Table 1**. Sixty-five percent of tumors were HR+/HER2-, 15% were HER2+, and 20% were TN. As expected, patients were younger in the TN subtype ($p = 1.18E-86$), the ductal type was more frequent in the HER2 + and TN subtypes ($p = 1.85E-40$), and grade 3 was more common in the TN subtype ($p < 2.2E-50$; **Table 1**). Expression levels of HER2 and TROP2 were heterogeneous across tumor samples with intensities spanning five intervals on the $\log_2$ scale (**Figure S3**). Compared with expression in NB samples, 34% of tumors were identified as TROP2-positive, and 22% as HER2-positive.

Tumor expression of both targets showed partial overlap, and double-positive tumors were observed. In the whole series (Fig. 2A), 32% of HER2-negative tumors were TROP2-positive and 20% of TROP2-negative tumors were HER2-positive. Thus, 47% of tumors were positive for TROP2 and/or HER2, including 9% that were double-positive, and 53% were double-negative. Combining anti-TROP2 and anti-HER2 ADCs could theoretically target 47% of tumors, corresponding to an absolute gain of 13%, and a relative gain of 39% compared with the largest percentage of tumors theoretically targeted with monotherapy (34% targeted by the anti-TROP2 ADC alone). This complementarity in HER2 and TROP2 expression was also observed in each molecular subtype and was more pronounced in the HR+/HER2- subtype than in the HER2 + and TN subtypes. The relative gain in tumors targeted by ADC combination therapy was 20% in the HR+/HER2- subtype, 6% in the HER2 + subtype and 10% in the TN subtype (Fig. 2B-D).

Altogether, these data revealed different degrees of complementarity and overlap in HER2 and TROP2 tumor expression and suggested that combining two ADCs should theoretically target a higher proportion of tumors than ADC monotherapy, both in the whole series and within each molecular subtype.

Gene expression of HER2 and TROP2 in cancer cells is complementary

We analyzed scRNAseq data from 206,347 epithelial cancer cells from primary BCs collected from 112 patients. All BCs but one (*in situ* ductal) were invasive; 58 were HR+/HER2-, 16 were HER2+, and 38 were TN. First, expression levels were heterogeneous across cancer cells, with a range of intensities spanning 4 to 5 intervals on the $\log_2$ scale (**Figure S4A**). Positive and negative expression was defined using the first quartile as the cut-off. When all cancer cells from the entire series were pooled, 29% were identified as HER2+, and 44% as TROP2+. As expected, we found more HER2 + cancer cells in HER2 + tumors (**Figure S4B**).

Second, we analyzed the degree of co-expression and complementary expression of the two targets at the cellular level. Analyzing pooling all cancer cells provided an idea of the global expression pattern of the two targets. Here too, the expression of both targets showed partial overlap; and double-positive cancer cells were observed. Across the entire series (Fig. 3A), 14% of cancer cells expressed HER2 only, 29% expressed TROP2 only, and 15% were double-positive. Among HER2- cells, 41% were TROP2+, and 21% of TROP2- cancer cells were HER2+, resulting in 58% of HER2 + and/or TROP2 + cancer cells and 42% that were double-negative. This complementarity in HER2 and TROP2 expression was observed in each molecular subtype (Fig. 3B-D). As expected, the percentage of double-positive cells was the highest in HER2 + tumors (32%) and the lowest in TN tumors (7%).

Third, we assessed the reproducibility of this complementarity in a *per-patient* analysis. The proportions of each cancer cell type in individual tumors are shown in Fig. 4. No tumor consisted exclusively of double-positive or double-negative cells. In the whole series, all but 3 tumors contained at least 3 different cancer cell types (Fig. 4A). Only 3% of tumors lacked double-positive cancer cells. *Per patient*, the median percentages of cancer cells were 48% (range, 3–98) for double-negative cells, 8% (range, 0–81) for HER2 single-positive cells, 23% (range, 0–92) for TROP2 single-positive cells, and 11% (range, 0–70) for double-positive cells. The results by molecular subtype are shown in Fig. 4B. In the HR+/HER2- subtype, the distribution of cancer cell types was very similar to that of the whole series. Conversely, in the HER2 + subtype, the median percentages of double-positive cells (27%) and HER2 single-positive cells (30%) increased compared with the whole series, while the median percentage of double-negative cells decreased (18%). In the TN subtype, the median percentage of double-negative cells (64%) increased, whereas the median percentage of double-positive cells decreased (5%). Altogether, these data on intra-tumor heterogeneity showed different degrees of complementarity and overlap in HER2 and TROP2 expression by cancer cells in BC samples.

ADC combination increases the number of targetable cancer cells

For each tumor, we measured the relative gain in targetable cancer cells achieved by the ADC combination *versus* “the best” ADC monotherapy. In the pooled cancer cells analysis, we observed a relative gain of 32% across the entire series (Fig. 3A). In individual tumors, the median relative gain was 13%, with strong variability (range, 0 to 85%; Fig. 5A). Figure 5B shows cancer cell proportions in one example of HR+/HER2- tumor, which had a relative gain of 41%. By molecular subtype, the median relative gain was 17% in HR+/HER2- tumors, 7% in HER2+, and 8% in TN (Fig. 5C).

Figure 5D shows the proportions of patients as a function of the relative gains in targetable cancer cells with ADC combinations. In the whole series, the proportions decreased as the relative gains increased, from 56% with a relative gain superior to 10%, to 30% with a relative gain superior to 20%, 20% with a relative gain superior to 30%, and 10% with a relative gain superior to 40%. These proportions varied by molecular subtype, being lower in the HER2 + subtype where HER2 + cancer cells are more prominent than in the HR + and TN subtypes.

Altogether, these data suggest that combining anti-HER2 and anti-TROP2 ADCs in BC patients should theoretically target a higher proportion of cancer cells than ADC monotherapy, both in the whole series and within each molecular subtype. The presence of double-positive cancer cells in nearly all tumors provides an opportunity to increase intracellular payload internalization with ADC combination. However, the percentage of double-negative cancer cells was relatively high, with a median close to 50% *per* patient in the whole population.

The complementarity in protein expression of HER2 and TROP2 in cancer cells increases the number of targetable cancer cells by ADC combination

We then assessed intra-tumor heterogeneity in protein expression of ADC targets by counting and analyzing the spatial organization of cancer cells stained with anti-HER2 and anti-TROP2 antibodies in eight invasive ductal BCs treated at our institution. These included 5 HR+/HER2- samples, 1 HR+/HER2+, and 2 TN. The general workflow of image analysis is shown in Fig. 6A. First, the proportions of each cancer cell phenotype (double-positive, simple-positive, and double-negative) for HER2 and TROP2 in individual tumors are shown in Fig. 6B. No tumor consisted exclusively of double-positive or double-negative cells. All tumors contained the four cell types in different proportions. *Per* patient, the median percentages of cancer cells were 27% (range, 3–64%) for double-negative, 19% (range, 2–31%) for HER2 single-positive, 10% (range, 3–41) for TROP2 single-positive, and 37% (range, 13–62%) for double-positive. The relative gain in targetable cells with ADC combination *versus* monotherapy ranged from 4% to 37% (Fig. 6B); 4 out of 8 patients (50%) had a value greater or equal to 10%, a percentage similar to the one observed with scRNAseq data.

Second, we analyzed the spatial distribution of the different cancer cell phenotypes in each tumor. For the single-positive cells (HER2 or TROP2), we observed two types of spatial organization (Fig. 6C, **top panel**): cells in niches, in which single-positive cells were surrounded by identical single-positive cells (“enriched region”), or an intermixed pattern, in which single-positive cells were intermingled with other cell populations (“heterotypic pattern”). The percentages of TROP2 or HER2 single-positive cells forming niches or showing an intermixed pattern are presented in Fig. 6C (**bottom panel**). The spatial structure of cell subpopulations varied considerably between samples, ranging from organization into well-defined niches to a largely intermixed distribution. In three of eight patients, more than 25% of TROP2 single-positive cells formed niches (dark pink bars), whereas in seven patients, more than 25% of HER2 single-positive cells formed niches (dark green bars). Heterogeneity in the spatial organization of the various cell phenotypes could also add to the potential of combining ADCs.

Altogether, these data confirmed at the protein level different degrees of complementarity and overlap in HER2 and TROP2 expression by cancer cells in patients with BC, as well as the potential for increasing payload entry and/or the number of targetable cancer cells through ADC combinations. They also revealed heterogeneous spatial organization of the different cancer cell phenotypes.

Discussion

Our study provides a preclinical rationale for combining anti-HER2 and anti-TROP2 ADCs in BC. We show that T-DXd combined with either Dato-DXd or S-Gov has synergistic antitumor activity in cell lines. This pharmacologic interaction is supported by complementary expression of HER2 and TROP2 across large cohorts of primary BCs, with marked intertumor and intratumor heterogeneity and frequent co-existence of HER2-only, TROP2-only, double-positive, and double-negative cancer cell populations within individual tumors. Interestingly, multiplex immunofluorescence reveals distinct spatial organization of target-positive cells. Together, these findings suggest that dual HER2/TROP2 targeting could broaden tumor-cell coverage within heterogeneous tumors, rather than simply increasing target engagement.

One major finding was the synergistic activity observed between anti-HER2 and anti-TROP2 ADCs in the three tested cell lines. Even lower concentrations of Dato-DXd and T-DXd in combination produced stronger antitumor effects than high concentrations of each drug alone. This observation confirms the synergistic effect of the T-DXd/Dato-DXd combination recently reported in three other HER2-overexpressing BC cell lines [34]. For our tested combinations, the strongest interactions occurred at intermediate, clinically achievable concentrations, arguing against a purely concentration-driven additive effect. Several non-mutually exclusive mechanisms may contribute to this synergy. Here, synergy was observed with both Dato-DXd and S-Gov, which target the same antigen but differ in molecular architecture and payload characteristics, thus supporting, at least in part, a mechanism related to target complementarity. Combining ADCs with different target antigens can contribute to the observed synergy. HER2 and TROP2 may provide partially independent routes for ADC internalization and intracellular payload delivery, thereby increasing the probability that a heterogeneous population of cancer cells receives a cytotoxic payload. In addition, the distinct pharmacologic properties of the ADCs used here (target-binding characteristics, linker properties, and payloads) may generate complementary patterns of intracellular drug exposure. For example, increased ADC internalization and efficiency were reported in double-positive BC cell lines and pre-clinical models with the T-DXd/Dato-DXd combination [34] or with the T-DM1/EV combination [45]. Similarly, Patritumab-Dxd (P-DXd), an anti-HER3 ADC, altered HER2 trafficking and enhanced T-DXd internalization in BC models [46]. Future studies should therefore determine whether and how synergy is preserved across additional ADC configurations.

Our large-scale, high-resolution transcriptomic analysis provides a complementary perspective by demonstrating that HER2 and TROP2 expression are neither redundant, nor mutually exclusive in BC. At the bulk-tumor level, a substantial proportion of tumors expressed either HER2 or TROP2, while a smaller subset coexpressed both targets. This observation was confirmed at the protein level in a series of 323 BC specimens [47] and a series of 244 samples of primary tumors and metastatic sites from 111 BC patients [48]. Altogether, these data support the rationale for dual targeting with ADCs, emphasizing that combining ADCs in tumors showing complementary expression of targets should theoretically increase the proportion of tumors that can be targeted compared with the most active single target. Importantly, this complementarity was observed across molecular subtypes, although its magnitude varied among them. The relative gain in targetable tumors was particularly evident in HR+/HER2 – tumors, whereas it was smaller in HER2 + tumors, which already represent a substantial targetable population with an anti-HER2 ADC.

However, bulk tumor analysis alone underestimates the complexity of Ag heterogeneity, particularly at the single-cell level. We therefore analyzed more than 200,000 epithelial cancer cells to reveal the marked heterogeneity in HER2 and TROP2 expression within individual BC tumors. Other scRNAseq studies of BC reported heterogeneity in HER2 and TROP2 gene expression in cancer samples [49] and circulating tumor cells [50], but did not quantify the complementarity in expression of the two targets. We observed that most tumors contained multiple cancer cell phenotypes in different proportions, including HER2-only, TROP2-only, double-positive, and double-negative populations. Similar observations were recently reported in small series of mesothelioma [51] and prostate cancer [52] with other surface targets (GFR/MET, and KLK2/STEAP1, respectively), and in ovarian carcinomas [53]. This finding is particularly relevant to ADC resistance because conventional biomarker classification is generally performed at the tumor level and may therefore obscure the coexistence of target-positive and target-negative cell clones within the same tumor. Residual target-negative cells can survive ADC treatment and provide a substrate for disease persistence and subsequent progression, representing one of the fundamental limitations of single-antigen ADC therapy. Combining HER2 and TROP2 ADCs could therefore increase the proportion of theoretically targetable cancer cells within individual tumors. Of note, the magnitude of this combination-related incremental gain varied considerably between patients, notably in the HR+/HER2- and TN subtypes. It was smaller in the HER2 + subtype. This interpatient variability suggests that dual-ADC therapy may not be uniformly beneficial. The coexistence of two phenomena, *i.e.* increase of targeted tumor cells and/or higher payload delivery in double-positive tumor cells, suggests a mechanistic framework in which dual ADC therapy could act through both the breadth of tumor cell coverage and the depth of payload delivery.

In addition, increasing the proportion of targetable cells is particularly relevant to the bystander effect, an important component of the activity of some ADCs. This effect rises with an increasing fraction of Ag-positive cells in a co-culture system, and with higher target expression levels on Ag-positive cells [54]. In HER2-heterogeneous tumors, therapeutic success depends on HER2 + cell abundance: tumors with an insufficient number of HER2 + cells may not internalize enough ADC to generate an excess of active payload capable of eliminating HER2- cancer cells *via* the bystander effect [10]. The actual benefit provided by the combination of ADCs may be greater than what is predicted by simply summing Ag+ cells. The bystander effect is all the more important because our protein-level analysis by mIF, although performed on a limited number of tumors, added crucial information about spatial organization. An association between ADC efficacy and the spatial distribution of target-positive cancer cells in tumors was reported in BC [5, 10]. We observed that single-positive cells were not necessarily randomly distributed; instead, they could appear either within enriched regions or in intermixed patterns. This distinction may be biologically and clinically relevant. A target-positive niche could represent a relatively homogeneous compartment that is efficiently exposed to one ADC, whereas an intermixed pattern may bring different target-positive populations into close proximity and could favor complementary targeting across neighboring cancer cells. Thus, spatial organization may influence how effectively a combination can cover the tumor as a whole. These hypotheses remain speculative. Nevertheless, this spatial heterogeneity supports the broader concept that ADC efficacy should ultimately be evaluated at the level

of the tumor ecosystem rather than solely as a binary biomarker measured in bulk tissue. Observations similar to ours were reported in bladder cancer with TROP2, Nectin-4 and HER2 [55]. .

Several limitations should be acknowledged. First, the synergy experiments were performed in a limited number of BC cell lines and therefore cannot capture the diversity of human tumors. Second, mechanistic studies are needed to understand synergy; experimental work directly measuring receptor occupancy, ADC internalization, intracellular trafficking, intracellular payload concentration, bystander effect, and cell killing according to HER2/TROP2 phenotype will be required. Third, mRNA expression cannot be assumed to be equivalent to functional surface-protein expression, although the positive relationship between transcript and protein levels across cancer cell lines provides supportive evidence. Fourth, the mIF analysis included only eight tumors, limiting our ability to draw conclusions about the prevalence or clinical significance of the spatial patterns observed. Finally, our analyses do not yet demonstrate that these properties translate into superior antitumor efficacy *in vivo* or into a clinically meaningful therapeutic window. These limitations underscore the need for validation in larger, spatially resolved cohorts and in models that reproduce tumor architecture and pharmacologic exposure. However, the convergence of pharmacologic, transcriptomic, single-cell, and spatial analyses provides a coherent rationale for dual Ag targeting, here limited to HER2/TROP2, but which can be extended to other target combinations.

This dual therapeutic strategy is a critical issue, as preclinical and clinical trials testing ADCs combinations are beginning to emerge. ADC combinations require careful consideration of the therapeutic index. HER2- and TROP2-directed ADCs, and other ADCs, can produce clinically meaningful toxicities [56], and their combination may increase cumulative adverse events. In preclinical studies, no significantly different toxicity was observed in xenografted mice treated with T-DXd/D-DXd [34], T-DM1/EV [45], and T-DXd/P-DXd [46] combinations compared with those treated with single ADCs. The first proof of concept in patients was demonstrated in the DAD phase I trial, which combined S-Gov with EV in urothelial cancers [32]. Grade 3 or higher adverse events were reported in 78% of patients, but no new safety signal with the ADC combination was observed compared with monotherapy in these heavily pretreated patients. Our expression analysis also provides a framework for biologically informed ADC combinations rather than supporting indiscriminate combination therapy. It suggests that conventional binary biomarker classification could eventually be complemented by quantitative measures of target heterogeneity, coexpression, and spatial organization. Based on our present results, we recently launched the ALTER clinical trial. ALTER is a French multicenter, randomized, open-label, phase II study assessing S-Gov/T-DXd combination in an alternating regimen for patients with advanced HER2-low TN BC eligible to S-Gov (NCT07151586). Patients are randomly assigned to the standard arm (S-Gov alone) or the experimental arm (S-Gov and T-DXd in an alternative scheme every two cycles). The hypothesis is that such upfront alternating regimen, before disease progression under S-Gov, may reduce the emergence of resistance and improve survival compared to S-Gov alone followed by T-DXd at progression. To our knowledge, it is the first trial of this type in BC.

In conclusion, our proof-of-concept study demonstrates that HER2 and TROP2 are complementary therapeutic targets across multiple levels of BC heterogeneity and that simultaneous targeting with anti-HER2 and anti-TROP2 ADCs can produce synergistic cytotoxicity *in vitro*. Prospective studies incorporating quantitative single-cell and spatial target profiling will be essential to determine whether target complementarity can serve as a predictive biomarker and, ultimately, whether rationally selected ADC combinations can overcome intratumor heterogeneity and improve clinical outcomes.

Declarations

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Table

Table 1: Clinicopathological characteristics of breast cancer samples used in the bulk transcriptome analyses.

Characteristics	All	HR+/HER2-	HER2+	TN	p-value*
Age at diagnosis, median year (range)	58 (22-96)	60.26 (24-96)	55.15 (21.93-93)	53.99 (24-96.29)	1.18E-86
Pathological type					1.85E-40
Ductal	6676 (80%)	4580 (77%)	985 (89%)	1111 (85%)	
Lobular	890 (11%)	798 (13%)	48 (4%)	44 (3%)	
other	805 (10%)	577 (10%)	70 (6%)	158 (12%)	
Pathological tumor size (pT)					7.80E-35
pT1	4204 (46%)	3201 (50%)	488 (40%)	515 (35%)	
pT2	4118 (45%)	2758 (43%)	595 (49%)	765 (52%)	
pT3	791 (9%)	451 (7%)	140 (11%)	200 (14%)	
Pathological axillary lymph node status (pN)					9.09E-18
Negative	5576 (59%)	4064 (60%)	584 (47%)	928 (60%)	
Positive	3938 (41%)	2673 (40%)	657 (53%)	608 (40%)	
Pathological grade					<1,00E-280
1	1241 (13%)	1172 (17%)	30 (2%)	39 (2%)	
2	4231 (43%)	3573 (53%)	390 (28%)	268 (15%)	
3	4406 (45%)	1984 (29%)	979 (70%)	1443 (82%)	

*, the p-value is for the Fisher's exact test

Figures

Figure 1

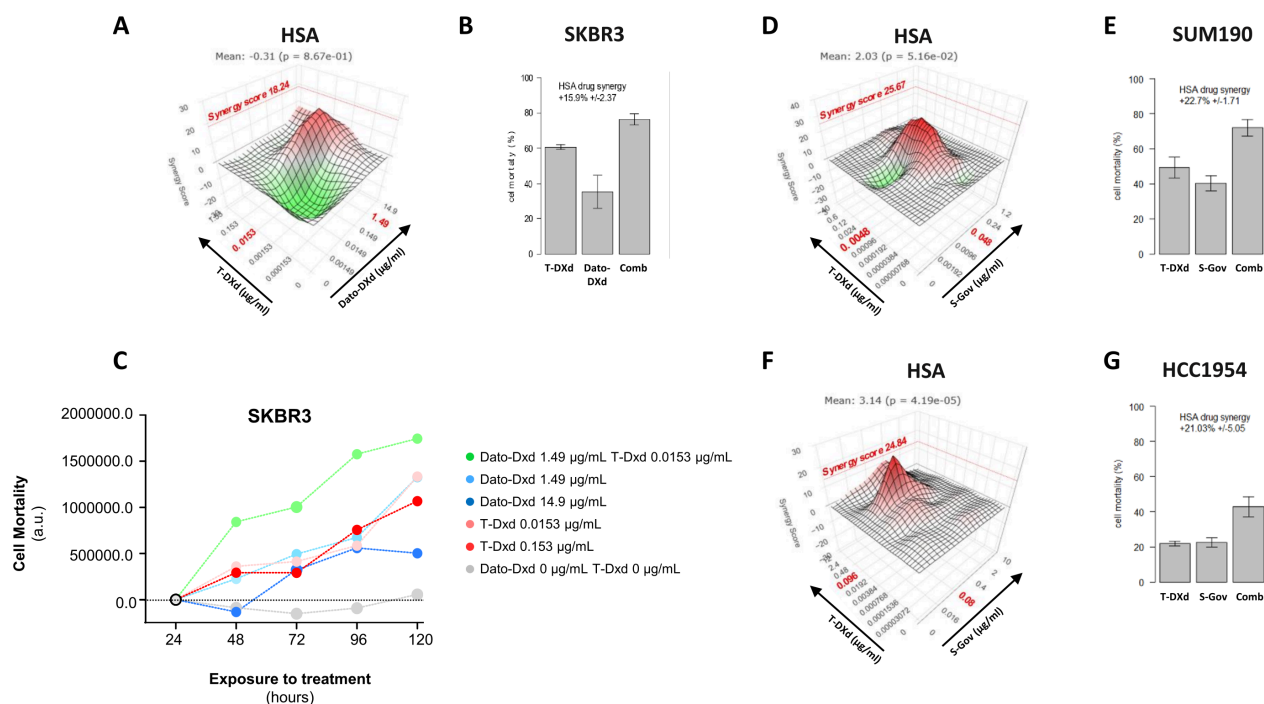


Figure 1

Figure 1: Synergistic effect of T-DXd and anti-TROP2 ADC combination in BC cell lines.

A/ Three-dimensional HSA synergy landscape for the combination of T-DXd and Dato-DXd in SKBR3 cell line. The 3D surface plot displays HSA synergy scores across the tested concentration matrix of T-DXd (Y-axis) and Dato-DXd (X-axis) (SG). The concentrations of the maximal HSA score are shown in red.

B/ Bar plot showing the cell death observed after 5 days of treatment for SKBR3, higher with ADC combination than with each single ADC used at identical doses.

C/ Monitoring of cell death overtime for SKBR3, based on analysis of data acquired using CellCiteX. The synergistic combination (in green) shows higher cell death overtime compared with identical doses of Dato-Dxd (in light blue), and T-Dxd (in pink) or doses 10 times higher (in dark blue and red, respectively) administered as single agents.

D/ Similar to A, but with SUM190 cell line and T-DXd and S-Gov.

E/ Similar to B, but with SUM190 cell line and T-DXd and S-Gov.

F/ Similar to D, but with HCC1954 cell line.

G/ Similar to E, but with HCC1954 cell line.

Figure 2

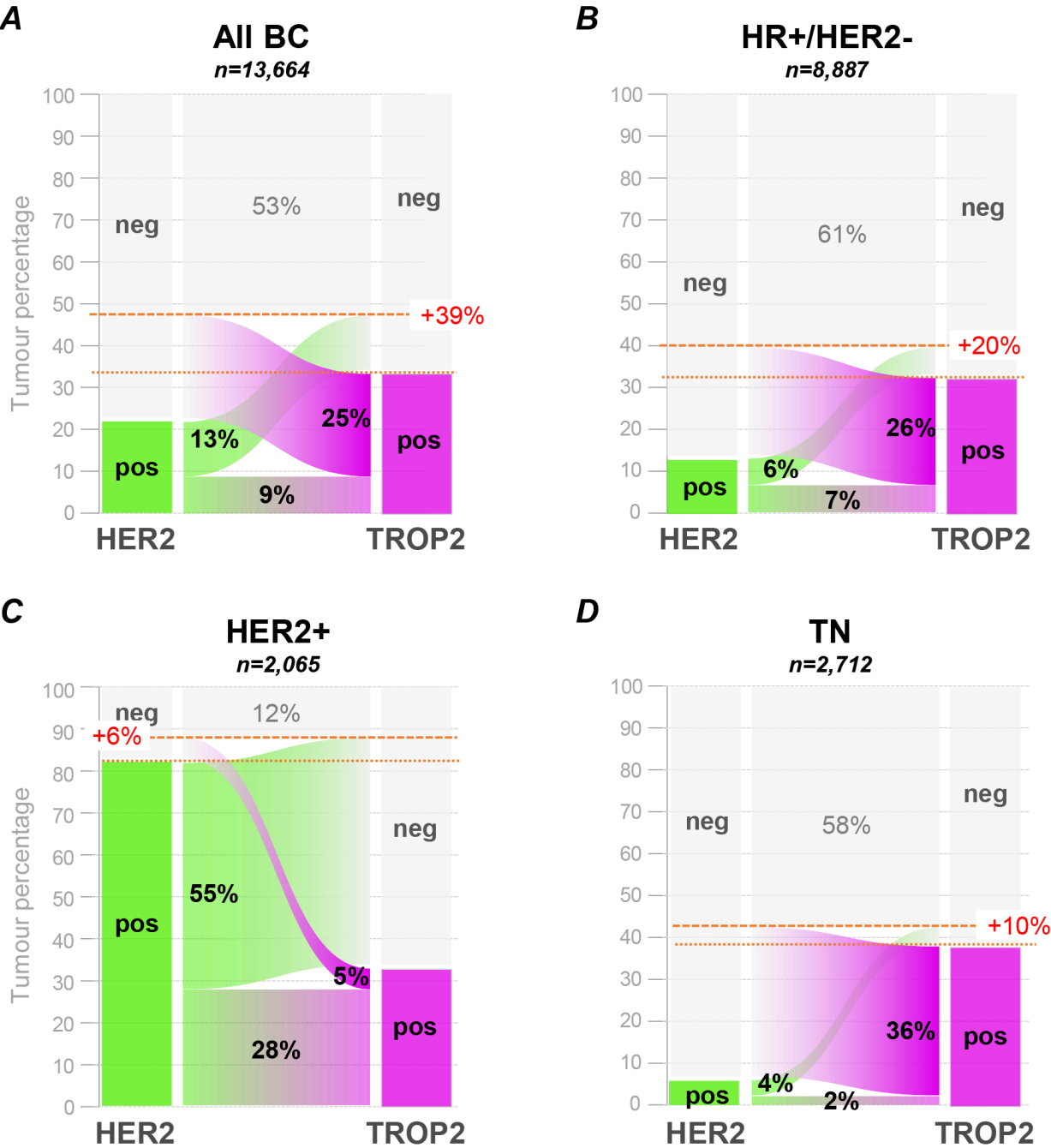


Figure 2

Figure 2: Intertumour heterogeneity: gene expression of HER2 and TROP2 in clinical BC samples at the bulk level.

A/ Alluvial Plot for gene expression status of HER2 and TROP2 in the whole population. The percentages of each of the four tumour groups are written in black (group positive for at least one gene) or grey

(group negative for both genes). Expression status of tumours is color-coded as follows: green for HER2-positive, pink for TROP2-positive, grey for negative, green-to-grey for HER2-positive/TROP2-negative, grey-to-pink for HER2-negative/TROP2-positive, green-to-pink for HER2-positive/TROP2-positive, and grey and grey for HER2- negative/TROP2-negative. The relative gain of tumours targeted by ADC combination *versus* “the best” ADC monotherapy is written in red.

B-D/ Similar to A, but in each molecular subtype: HR+/HER2- (B), HER2+ (C), and TN (D).

Figure 3

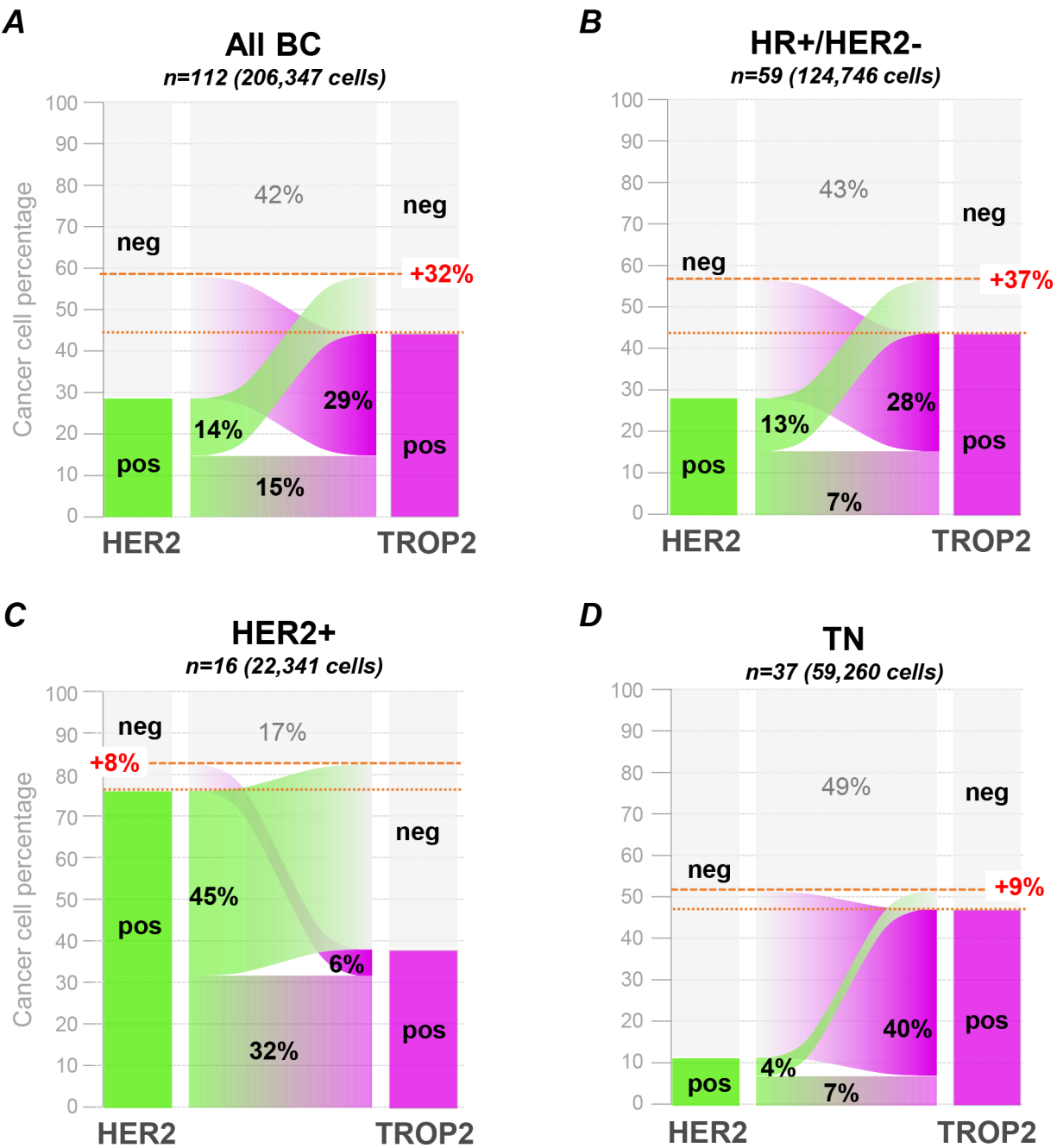


Figure 3

Figure 3: Intratumour heterogeneity: gene expression of HER2 and TROP2 in clinical BC samples at the single-cell level.

A/ Alluvial Plot for gene expression status of HER2 and TROP2 targets in the pooled cell population of the whole series. The percentages of each of the four cancer cell types are written in black (group positive for at least one gene) or grey (group negative for both genes). Expression status of cancer cells is color-coded as follows: green for HER2-positive, pink for TROP2-positive, grey for negative, green-to-grey for HER2-positive/TROP2-negative, grey-to-pink for HER2-negative/TROP2-positive, green-to-pink for HER2-positive/TROP2-positive, and grey and grey for HER2- negative/TROP2-negative. The relative gain of cancer cells targetable by ADC combination *versus* “the best” ADC monotherapy is written in red.

B-D/ Similar to A, but in each molecular subtype: HR+/HER2- (B), HER2+ (C), and TN (D).

Figure 4

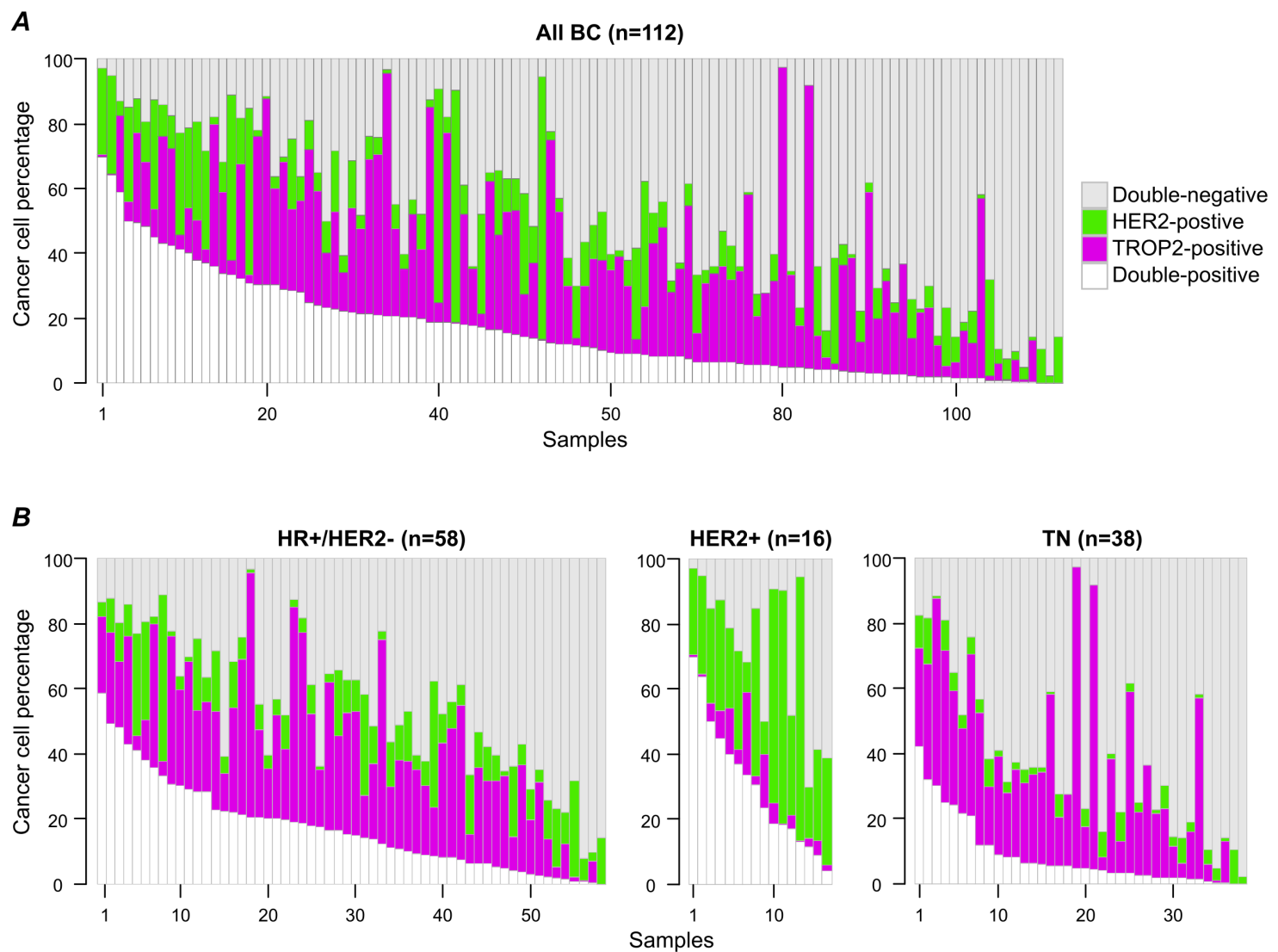


Figure 4

Figure 4: Intratumour heterogeneity: proportion of each cancer cell type according to HER2 and TROP2 expression in individual BC patients.

A/ In the whole series (N=112 patients). Expression status of cancer cells is color-coded as follows: green for HER2-positive/TROP2-negative, pink for HER2-negative/TROP2-positive, white for double-positive, and grey for double-negative. The samples are ordered from left to right by decreasing percentage of double-positive cancer cells.

B/ Similar to A, but in each molecular subtype: HR+/HER2-, HER2+, and TN.

Figure 5

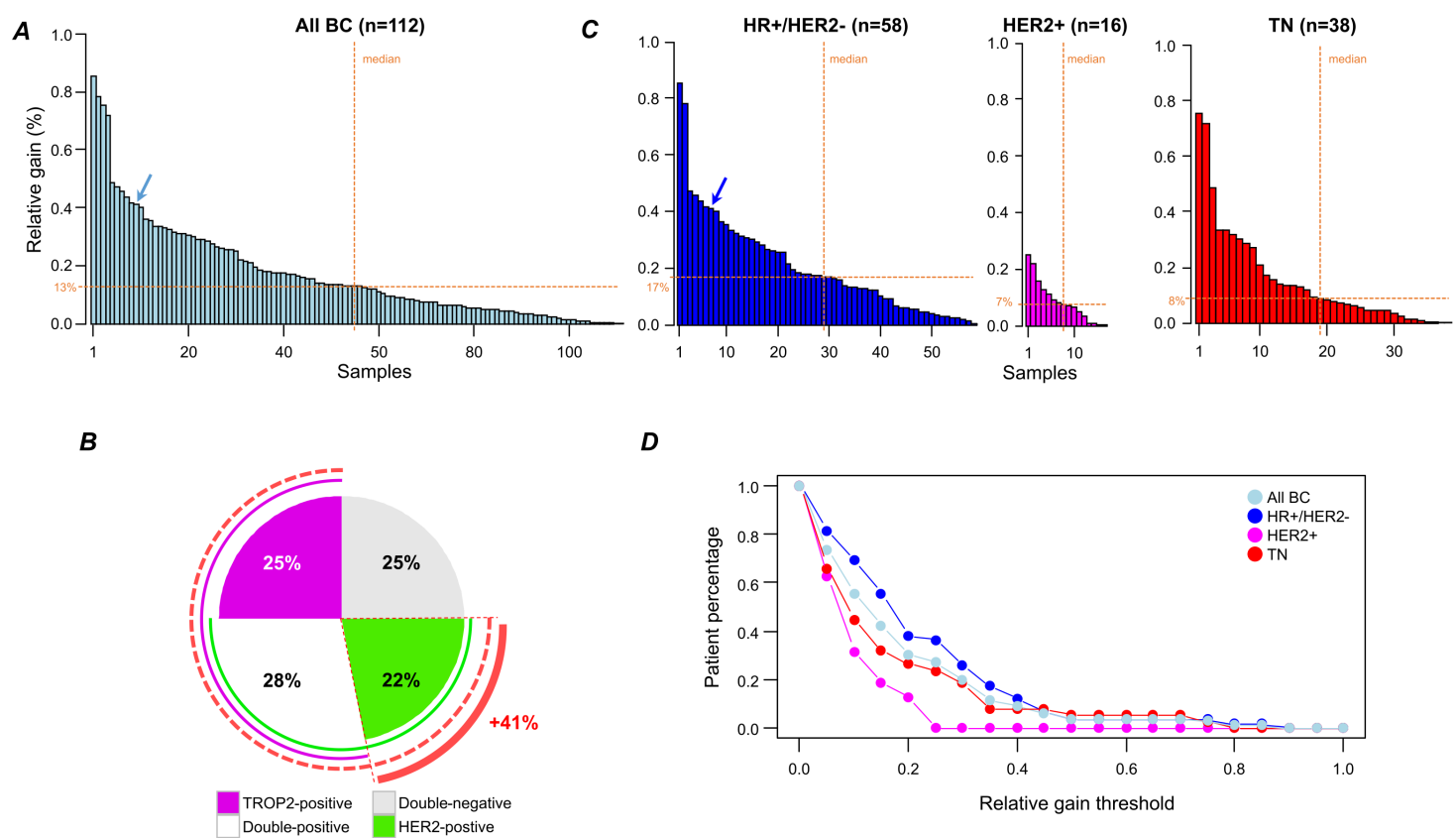


Figure 5

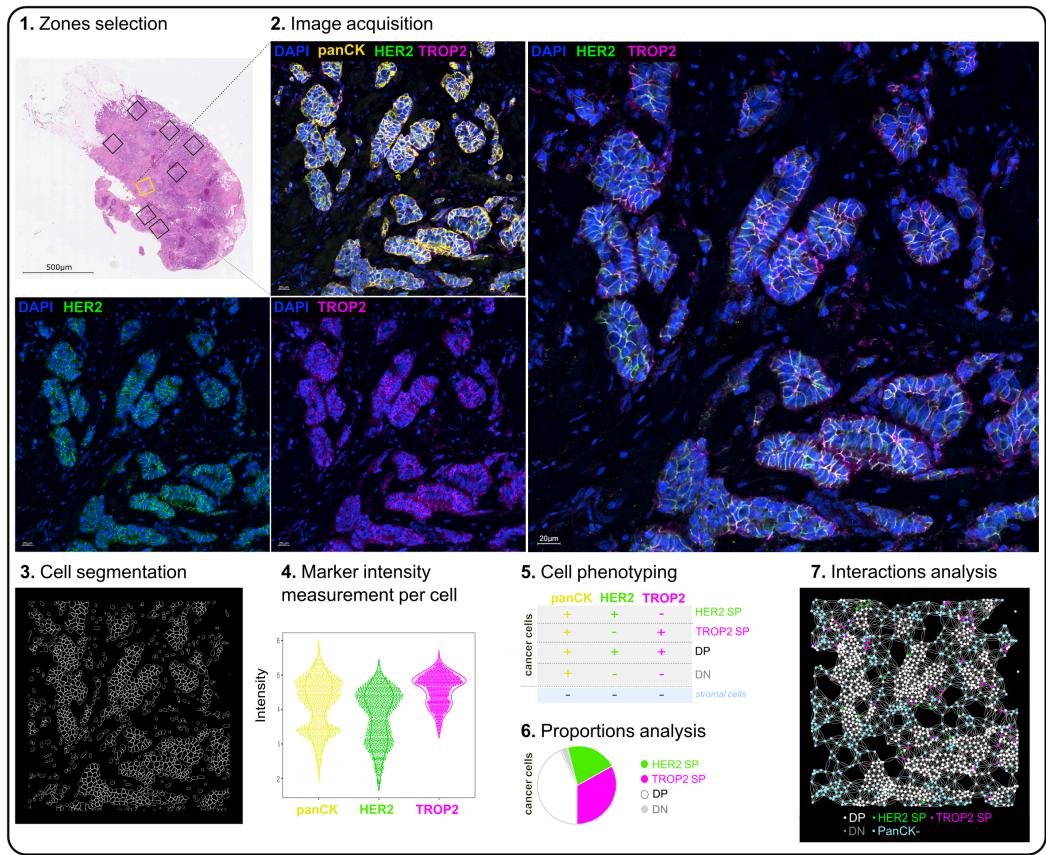
Figure 5: Anti-HER2 and anti-TROP2 ADCs combination increases the number of targetable cancer cells.

A/ Relative gain of targetable cancer cells by ADC combination in each individual patient in the whole series (N=112). The samples are ordered from left to right by decreasing relative gain. The blue arrow indicates the tumor shown in (B).

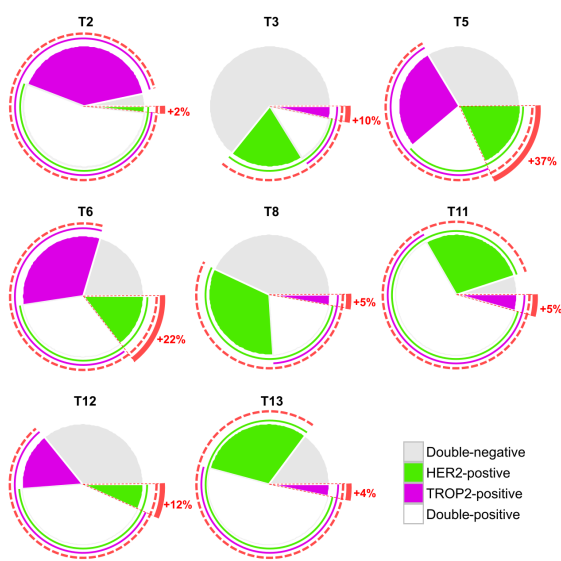
B/ Pie chart showing the proportions of the different cancer cells types according to HER2 and TROP2 expression in one HR+/HER2- tumour with a relative gain of 41%. Expression status of cancer cells is color-coded as indicated. The green circular arc represents the cancer cells targetable by anti-HER2, the pink circular arc represents the cancer cells targetable by anti-TROP2, the dashed red circular arc represents the cancer cells targetable by anti-HER2 and anti-TROP2 combination, and the solid red circular arc represents the relative gain in cancer cells targetable by anti-HER2 and anti-TROP2 combination. C/ Similar to A, but in each molecular subtype.

D/ Proportions of patients as a function of the relative gains in targetable cancer cells by ADC combination, in the whole series (black curve), and in each molecular subtype (HR+/HER2-: blue; HER2+: purple; TN: red).

A. Image analysis workflow



B. Proportions of cancer cells



C. Spatial organization of cancer cells

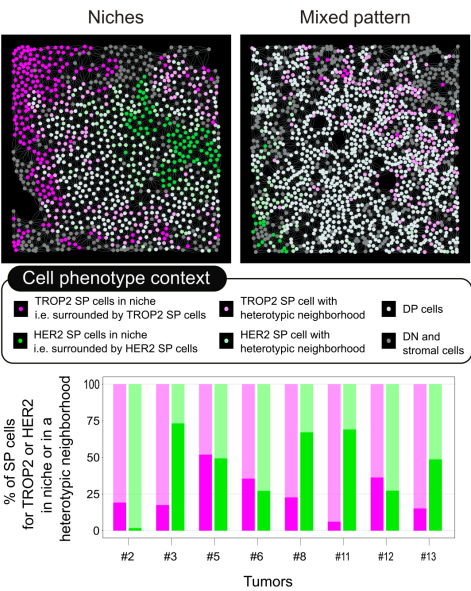


Figure 6

Figure 6: Intratumour heterogeneity: protein expression of HER2 and TROP2 in clinical BC samples at the single-cell level.

A/ Work-flow of analyses. Images of multiplex IF are shown in (2) and show HER2 and TROP2 protein expression on cancer membrane as green and pink signals respectively. HER2+/TROP2- cells,

HER2-/TROP2+ cells, double-positive cells and double-negative cells are indicated.

B/ Pie charts showing the proportions of the different cancer cells types according to HER2 and TROP2 expression in eight BCs stained by multiplex IF. Expression status of cancer cells is color-coded as indicated. The green circular arc represents the cancer cells targetable by anti-HER2, the pink circular arc represents the cancer cells targetable by anti-TROP2, the dashed red circular arc represents the cancer cells targetable by anti-HER2 and anti-TROP2 combination, and the solid red circular arc represents the relative gain in cancer cells targetable by anti-HER2 and anti-TROP2 combination.

C/ Spatial analysis. *Top*; Cell maps showing the distribution of cancer cells in one tumor according to their phenotype and spatial interactions. Interactions between cells are defined by Delaunay triangulation. Cells are color-coded as follows: light pink for TROP2-positive cells interacting with at least one HER2-positive or double-positive cell, dark pink for TROP2-positive cells not interacting with HER2-positive or double-positive cells, light green for HER2-positive cells interacting with at least one TROP2-positive or double-positive cell, dark green for HER2-positive cells not interacting with HER2-positive or double-positive cell, white for double-positive cells, and grey for double-negative cells and stromal cells. Double arrows between cells denote a spatial interaction: on le voit pas. *Bottom*, bar plots showing for each tumor the percentage of TROP2-positive cells in niche or surrounded by TROP2-positive cells and the percentage of HER2-positive cells in niche or surrounded by HER2-positive cells.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [FigureS1.png](#)
- [FigureS2.png](#)
- [FigureS3.png](#)
- [FigureS4.png](#)
- [TableS1.xlsx](#)
- [TableS2.xlsx](#)